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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 April 2002 (18.04.2002)

PCT

(10) International Publication Number
WO 02/31510 A1

(51) International Patent Classification[7]: G01N 33/68, C07K 1/04

(21) International Application Number: PCT/NL01/00744

(22) International Filing Date: 10 October 2001 (10.10.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
00203518.6 11 October 2000 (11.10.2000) EP

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/31510 A1

(54) Title: IDENTIFICATION OF PROTEIN BINDING SITES

(57) Abstract: The invention relates to the field of molecular recognition or detection of discontinuous or conformational binding sites or epitopes corresponding to a binding molecule, in particular in relation to protein-protein protein-nucleic acid, nucleic acid-nucleic acid or biomolecule-ligand interactions. The invention provides a synthetic molecular library allowing testing for, identification, characterisation or detection of a discontinuous binding site capable of interacting with a binding molecule, said library having been provided with a plurality of test entities, each test entity comprising at least one first segment spotted next to a second segment, each segment having the capacity of being a potential single part of a discontinuous binding site.

IDENTIFICATION OF PROTEIN BINDING SITES

The invention relates to the field of molecular recognition or detection of discontinuous or conformational binding sites or epitopes corresponding to or interacting with a binding molecule, in particular in relation to protein-protein or protein-ligand interactions.

Interactions between binding molecules, which in general are biomolecules, and their corresponding ligands, are central to life. Cells often bear or contain receptor molecules that interact or bind with a hormone, a peptide, a drug, an antigen, an effector molecule or with another receptor molecule; enzymes bind with their substrate; antibody molecules bind with an antigen, nucleic acid with protein, and so on. By "interact or bind" it is meant that the binding molecule and ligand approach each other within the range of molecular forces, and may influence each others properties. This approach takes the binding molecule and its ligand through various stages of molecular recognition comprising increasing degrees of intimacy and mutual effect: they bind.

Binding molecules have this binding ability because they comprise distinct binding sites allowing for the recognition of the ligand in question. The ligand, in turn, has a corresponding binding site, and only when the two binding sites can interact by -- essentially spatial -- complementarity, the two molecules can bind. Needless to say that, molecules having three dimensions, binding sites are of a three dimensional nature, often one or more surface projections or protuberances of one binding site correspond to one or more pockets or depressions in the other, a three-dimensional lock-and-key arrangement, sometimes in an induced-fit variety.

Sometimes, such a protuberance comprises a single loop of the molecule in question, and it is only this protuberance that essentially forms the binding site. In that case one often terms these binding sites as comprising a linear or continuous binding site, wherein a mere linear part of the molecule in question is in essence responsible for the binding interaction. This terminology in widely used to describe for example antibody-antigen reactions wherein the antigen comprises part of a protein sequence,

a linear peptide. One than often speaks about a linear or continuous epitope, whereby the binding site (epitope) of the antigenic molecule is formed by a loop of consecutively bound amino acids. However, similar continuous binding sites (herein epitope and binding site are use interchangeably) can be found with receptor-antigen interactions (such as with a T-cell receptor), with receptor-ligand interactions such as with hormone receptors and agonists or antagonists thereof, with receptor-cytokine interactions or with for example enzyme-substrate or receptor-drug interactions, whereby a linear part of the molecule is recognised as the binding site, and so on.

More often, however, such a protuberance or protuberances and depressions comprise various, distinct parts of the molecule in question, and it are the combined parts that essentially form the binding site. Commonly, one names such a binding site comprising distinct parts of the molecule in question a discontinuous or conformational binding site or epitope. For example, binding sites laying on proteins having not only a primary structure (the amino acid sequence of the protein molecule), but also secondary and tertiary structure (the folding of the molecule into alpha-helices or beta-sheets and its overall shape), and sometimes even quaternary structure (the interaction with other protein molecules) may comprise in their essential protuberances or depressions amino acids or short peptide sequences that lay far apart in the primary structure but are folded closely together in the binding site.

Due to the central role binding molecules and their ligands play in life, there is an ever expanding interest in testing for or identification of the nature or characteristics of the binding site. Notably the rapid developments in evolving biotechnology fields such as proteomics will result in the near future in the identification of more and more binding molecules and their corresponding ligands; the detection of protein-protein interactions, but also of enzyme-substrate interactions (not only of protein enzymes but certainly also of for example catalytic RNA-based interactions), and the identification of protein-nucleic acid and of nucleic acid-nucleic acid pairs of binding molecule and corresponding ligand, will certainly result in generating more interest in where the exact interacting (binding) sites between these molecules lay, and how one can develop compounds (agonists, antagonists, drugs) modulating the specific interaction.

Not only is one interested in the exact nature of the particular interaction between binding molecule and ligand in question, for example in order to replace or supplement binding molecules or ligands when needed; one is also interested in knowing approximating characteristics of the interaction, in order to find or design analogues, agonists, antagonists or other compounds mimicking a binding site or ligand involved.

Versatile and rapid methods to test for or identify continuous epitopes or binding sites are known. Most, if not all nucleic acid detection techniques, and molecular libraries using these, entail hybridisation of an essentially continuous nucleic acid stretch with a complementary nucleic acid strand, be it DNA, RNA or PNA. Little attention has been paid to methods allowing rapid and straightforward identification of discontinuous binding sites of an essentially nucleic acid nature. Although plenty of such sites exist, think only of the lack of understanding surrounding ribosomal binding sites where ribosomal proteins bind to tRNA, of regulatory sites in promotor sequences, of interactions between polymerases and replicases between DNA and RNA, of catalytic RNA reactions, and so on, no molecular libraries exist that provide easy access to such sites.

An early work in the peptide field is WO 84/03564, related to a method of detecting or determining antigenically active amino acid sequences or peptides in a protein. This work, providing the so-called Pepscan technology, whereby a plurality of different peptides is synthesised by linking with a peptide bond a first amino acid to a second, and so on, and on a second position in the test format yet another first amino acid is linked to a second, and so on, after which the synthesised peptides are each tested with the binding molecule in question, allows the determination of every continuous antigenic determinant or continuous epitope of importance in a protein or peptide sequence. Pepscan technology taken in a broad sense also provides for the testing for or identification of (albeit linear) peptides essentially identical with, analogous to or mimicking binding sites or ligands of a various nature (mimotopes, Geyssen at al, Mol. Immunol. 23:709-715, 1986).

Pepscan technology allows identification of linear peptide sequences interacting with receptor molecules, enzymes, antibodies, and so on, in a rapid and straightforward fashion, allowing testing a great many peptides for their reactivity with the binding molecule in question with relatively little effort. The order of magnitude of testing

capability having been developed with Pepscan technology (e.g. also due to miniaturisation of test formats, see for example WO 93/09872) furthermore allows at random testing of a multiplicity of peptides, leading to automated combinatorial chemistry formats, wherein a great many of binding molecules are being tested in a (if so desired at random) pattern for their reactivity with a molecular library of synthetic peptides representing potential continuous binding sites or ligands, allowing the rapid detection of particularly relevant molecules out of tens of thousands of combinations of molecules tested.

However, for the testing of discontinuous or conformational binding sites to a binding molecule, no formats similar to or as versatile as Pepscan technology exist. Attempts to identify discontinuous epitopes by Pepscan technology are cumbersome. It does in general not suffice to merely extend synthesis of the test peptides by linking more amino acids to the existing peptide, and hoping that some of the thus formed longer peptides will fold in such a way that at least two distinct parts are presented in a discontinuous fashion and are recognised by a binding molecule. Than there is no way of finding out in a rapid and straightforward fashion that the binding is indeed through a discontinuous binding site, it might be that just a longer single loop is responsible for the binding.

Some additional possibilities are provided by testing synthetic peptide sequences that have been designed to comprise two previously identified parts of a binding site, each part in essence being linear and being part of a larger linear peptide. Early work herein was done by Atassi and Zablocki (J. Biol. Chem 252:8784, 1977) who describe that spatially or conformationally contiguous surface residues (which are otherwise distant in sequence) of an antigenic site of egg white lysozyme were linked by peptide bonds into a single peptide which does not exist in lysozyme but attempts to simulate a surface region of it. However, their technique, called surface simulation synthesis, requires the detailed knowledge of the three-dimensional structure of the protein under study and a full chemical identification of the residues constituting the binding site at beforehand, as well as their accurate conformational spacing and directional requirements.

In the same fashion, Dimarchi et al (Science 232:339-641, 1986) describe a 38 to 40 amino acid long synthetic peptide consisting of two previously identified separate peptidyl regions of a virus coat protein. The peptide was synthesised using common

peptide synthesis technology (Merrifield et al., Biochemistry 21, 5020, 1982) by adding subsequent amino acids with a peptide bond to an ever growing peptide resulting in a peptide wherein the two peptidyl regions were connected by a diproline spacer presumably functioning as indication of a secondary structural turn, thereby thus providing a two-part epitope or binding site.

However, it is clear that when one already at beforehand has to know the sequence of the (in this case only) two relevant parts, in order to provide the desired discontinuous binding site, it excludes the feasibility to provide (desirably in a random fashion) a whole array of merely potential discontinuous binding sites for large scale testing. Furthermore, a major drawback of the above mentioned strategies is that again only linear epitopes or dominant binding regions of discontinuous eptitopes can be mimicked adequately. For the more complete synthesis of a discontinuous binding site, all the contributing parts have to be arranged in the proper comformation to achieve high-affinity binding, therefore, single parts of discontinuous binding sites have to be linked.

Fifteen years after Dimarchi, Reineke et al (Nature Biotechnology, 17:271-275, 1999) provided a synthetic mimic of a discontinuous binding site on a cytokine and a method to find such a discontinuous binding site that allowed for some flexibility and somewhat larger scale of testing, wherein positionally addressable peptide collections derived from two separate regions of the cytokine were displayed on continuous cellulose membranes, and substituted in the process to find the best binding peptide. After selection of the "best reactors" from each region, these were combined to give rise to another synthetic peptide collection (comprising peptides named duotopes) that again underwent several rounds of substitutions.

Reineke et al hereby provide synthesis of peptide chains comprising duotopes, however, again selected after previous identification of putative constituting parts with Pepscan technology, thereby still not allowing testing discontinuous binding sites in a rapid and straight forward fashion.

However, as indicated before, protein domains or small molecules that mimic binding sites are playing an increasing role in drug discovery, diagnostics and biotechnology. The search for particular molecules that bind to a binding site and mimic, or antagonise the action of a natural ligand has been initiated in many laboratories. As indicated before, attempts to find such structures in synthetic molecular libraries

often fail because of the essentially discontinuous nature and spatial complementarity of most binding sites. Thus, for the many more cases where the binding site may essentially be discontinuous improved means and methods to identify these sites are needed, and in particular means and methods are needed that allow testing for discontinuous binding sites whereby said parts need not necessarily first be selected by previous identification as a putative or even only tentative constituting part of the desired discontinuous binding site, but bear only the potentiality of being part of that site by being a molecule with more or less distinct features per se.

The invention provides a method for producing a molecular library comprising providing said library with a plurality of test entities wherein said entities have essentially been produced by segment spotting, that is by spotting, placing, or attaching in close proximity at least two (di-, tri, oligo- or multimeric) segments of for example nucleic acids or peptides directly or indirectly to a solid phase, such as an array surface, instead of by sequentially synthesising test molecules and spotting one molecule, or several replicas of said one molecule, as a single entity, which is done traditionally. In theory, said segments can be sequentially synthesised in close proximity to each other, whereby in a repetitive fashion, one monomer (e.g. a nucleotide or an amino acid) to another, until a (in essence polymeric) molecule (segment) of the desired length has been obtained. Essentially, existing nucleic acid libraries comprise nucleic acids that are synthesised sequentially, by adding one nucleotide or nucleoside at a time to the growing stretch, and existing peptide libraries comprise peptides that are synthesised sequentially, by adding one amino acid at the time to a growing stretch, until the desired length has been reached, however, with existing libraries, no attention is given to synthesising specific segments in close proximity to each other, so that they together can represent a putative binding site. With nucleic acids said monomers are essentially selected from a limited set of well known nucleotides, with peptides, said monomers are essentially selected from a well known set of amino acids. Not only naturally occurring monomers are used, synthetic nucleotides, such as peptide nucleic acid (PNA) molecules, or non-naturally occurring amino acids, or even D-amino acids, are routinely used as monomers by which the essentially polymeric molecules are

generated or produced, using a method that is essentially conform the sequential synthesis of polymers from monomeric molecules in nature. Preferred, according to the invention, however, is synthesising the segments before they are attached to the solid phase in close proximity, thereby it is easier to create the desired test entity, the putative binding site composed of two or more segments located in close proximity and attached to the solid phase, e.g. the array surface. In close proximity herein reflects the possibility that a putative binding molecule can bind to at least two of the closely spotted segments or parts thereof, and is defined in angstrom units, reflecting the in general molecular scale of the binding sites, it is preferred to attach the two or more segments that form the desired test entity at no more than 100 angstrom away from each other, however, obviating the need of long linkers, or when small segments are used, in between distances of smaller than 50, or preferably smaller than 30, or even smaller than 15 angstrom are preferred, said smaller distances in general creating a better fit for binding sites. Minimal proximity is 1-2 angstrom, whereby the segments are for example linked to variously protected thiol groups only 1-2 atoms on the polymer away from each other. Furthermore, the length of a flexible linker should preferably be 10-100 angstrom, where the preferred length of segments is at about 5-100 angstrom, where the preferred distance between the tops of segments amounts to 0-30 angstrom.

For example, two segments can be coupled, preferably as loops, onto a (polycarbon)-polymer surface. With extra spaced building blocks (for example phenylalanine amino acids) it is provided to obtain extended loops. On the (polycarbon)-surface for example two types (see for suitable types also fig. 1.) of protected cysteines (e.g. cys (trt) and cys (mmt)) and for example one spacing building block is coupled. The cys (mmt) is deprotected with 1% TFA while the cys (trt) remains protected. The first segment is coupled to the deprotected cys (mmt). Then the second cys (trt) is deprotected with 95% TFA. Then the second segment is coupled to the now deprotected cys (trt). If desired, segments can also be linked together, using appropriate chemistry.

Alternatively, instead of directly linking the segments to said surface (albeit via linkage groups), said segments may be first linked to a template that in itself is linked to the surface. In a preferred embodiment, such as template is for example a peptide. For example, two segments can be coupled onto a cyclic template that itself

is coupled to the polymer surface. The cyclic template is for example a cyclic flexible peptide. The cyclic peptide contains for example reactive groups such as four lysines (mmt), two cysteines (trt) and two cysteines (butyl). The template is for example coupled to the resin via a sulphur. The invention thus provides a molecular library that, albeit also suited for detecting or screening for continuous binding sites, is now particularly well suited for detecting or screening for discontinuous binding sites, in particular in relation to binding molecule-ligand interactions such as for example protein-protein, protein-nucleic acid, and nucleic acid-nucleic acid interactions, now that at least two different segments, each of which may represent a part of a discontinuous binding site, are spotted as single entity, tentatively representing a, possibly as yet unknown, discontinuous binding site, herein also called a binding body.

Within this description, the term binding body is generally used for essentially all-peptide segment constructs, however, the technology, as described for all-peptide combinations can of course also be used for nucleic acid combinations or combinations of an even more mixed nature. A binding body, which is in essence a synthetic molecule comprising a binding site identifiable or obtainable by a method according to the invention as described herein, is essentially a combination of random peptide segments (fixed into one molecule or represented as one molecule on a test surface) which acts as an binding molecule such as an antibody. Just as in the case of antibodies, the recognition may more or less be "degenerate" e.g. the binding site on the target molecule need not always be optimal. The binding body may in principle bind to any part of the target molecule. For instance: to neutralise the action of TNF-alfa one might develop a small molecule that specifically interacts with the receptor binding site on TNF-alfa; alternatively one might develop an antibody that interacts with TNF-alfa at an as yet undefined place and neutralises its action. This shows that sometimes small molecules are the solution and sometimes large antibodies. Unfortunately both have their disadvantages: small molecules are difficult or impossible to make for large recognition sites, large molecules like antibodies are much easier to develop however cannot be used intracellularily and have all sorts of pharmacological disadvantages like their immunogenicity and their inability to act inside the cell.

The advantageous properties of the binding body combine those of small and large molecules; binding bodies share advantages of both. A preferred binding body consists of random peptide segments for example slightly biased or shuffled to resemble CDR's or other binding domains. If needed or desired, CDR's may be mimicked by using for example 6 segments, each representing one possible CDR, however, combinations of 2, 3 or 4 segments will already provide diversity. The peptide segments are linked at, preferably, both sides to a scaffold or solid phase. Thus binding bodies are made up of molecules with one, two or more peptide segments. Highly diverse binding body libraries can be generated based on systematic combination of relatively small numbers of random peptide segments. A library of 100 binding bodies is easily produced using positional defined peptide segment array's as described in this application. Screening of such a library with any given molecule is simple, fast and straightforward. Hits can be translated directly into the amino acid or segment make up of the binding body (due to the positional defined array). A library of 10,000 binding bodies can be easily generated by combining all peptides from smaller libraries with each other, or starting with a larger solid support surface. A library of 1,000,000 binding bodies can for example be easily generated by combining all peptides of smaller libraries into binding bodies that contain three segments. Thus a large diversity of binding bodies can be generated starting with relatively small numbers of random peptides (for instance 10) and multiple combinations of peptides combined into a single binding body (for instance 6) to arrive at a diversity of 1,000,000 or even larger. Alternatively the same binding body diversity can be obtained starting with for example 1000 random peptides and using just two peptide segments for each binding body. Just as antibodies, binding bodies can "mature" . Based on hits obtained with an initial set of random binding bodies (above) new dedicated libraries can be generated that will contain a high number of improved combinations. The best ones can be selected or improved in an additional round using a second dedicated library, and so on. Development of high affinity binding bodies is thus provided by chemistry to bind peptides with, preferably both end's, to a molecular scaffold or solid phase, by using an array system in which each binding body is positionally defined, further by appropriate miniaturisation and/or by appropriate bioinformatics to analyse the data and to design subsequent improved binding bodies or dedicated libraries of binding bodies.

Said two or more different segments can of course each be selected at random from any set of di-, tri,- or oligomeric sequences, such as from di-, tri,- or oligonucleotides, or di-, tri-, or oligopeptides, but sometimes it may be preferred to include at least one specific segment in said entity, specific in a sense that it has been selected from among known segments or distinct parts of biomolecules, such as parts of genes, proteins, enzymes, nucleic acids or unique fragments thereof, proteins involved in up- or downregulation of translation, t-RNAs, SNRP's, antibodies, complementarity determining regions (CDR's), antigens, receptors, transport proteins, transcription factors or factors involved in up- or downregulation of transcription, promotor sequences such as but not necessarily restricted to the well known TATA-box elements, repressor sites, operator sites and other control elements, polymerases, replicases, in short, from among known segments or distinct parts of binding molecules known or suspected to be involved in binding via a discontinuous binding site.

Known segments or parts thereof spotted in close proximity may of course be already known as parts constituting a discontinuous binding site, however, previous identification as such is in essence not necessary, since screening for such sites with a molecular library according to the invention allows rapid and straightforward identification of said constituting segments or parts thereof.

Screening such a library can easily be envisioned when the library's molecules differ only in that constituting segments are chosen in an overlapping fashion, whereby a first segment from a distinct biomolecule is spotted next to a second, and to a third, and to a fourth segment, and a second is spotted next to a third, and to a fourth, and so on, if so required until all possible segments of said biomolecule have been spotted in close proximity two-by-two (or three-by-three, or even more) together, which allows for a systematic screening of possible discontinuous binding sites present on said biomolecule.

However, an overlapping fashion is of course not required, random segment combinations spotted in close proximity will provide valuable information about binding sites as well.

The invention thus provides a method for producing a molecular library for identification or detection of a binding site capable of interacting with a binding molecule, and thus for the identification of a molecule as a binding molecule, said

method comprising providing said library with a plurality of segments derived from binding molecules or their ligands, further comprising spotting at least two of said segments in a pair, or three in a threesome, or more in the respective plurality, preferably a greater part of said pairs, threesomes or pluralities, most preferably essentially all of said pairs, threesomes or pluralities, by at least spotting a first segment next to a second segment, for example a segment which comprises a dimer, trimer, oligomer or multimer.

Existing libraries, be it of for example nucleic acid (containing a repetitive back-bone of nucleotides, nucleosides or peptide nucleic acid, or combinations of these) or amino acid (containing a repetitive back-bone of amino acids) nature have in general in common that single molecules (or single segments) or a plurality of replicas of said single molecules are spotted an used as the entity representing the binding site. Such libraries comprise oligomeric or multimeric molecules, such as stretches of nucleic acids or amino acids, that have been produced by sequentially linking, in a repetitive fashion, one monomer (e.g. a nucleotide or an amino acid) to another, until a (in essence polymeric) molecule of the desired length has been obtained. Essentially, existing nucleic acid libraries comprise nucleic acids that are synthesised sequentially, by adding one nucleotide or nucleoside at a time to the growing stretch, and existing peptide libraries comprise peptides that are synthesised sequentially, by adding one amino acid at the time to a growing stretch, until the desired length has been reached. With nucleic acids said monomers are essentially selected from a limited set of well known nucleotides, with peptides, said monomers are essentially selected from a well known set of amino acids. Not only naturally occurring monomers are used, synthetic nucleotides, such as peptide nucleic acid (PNA) molecules, or non-naturally occurring amino acids, or even D-amino acids, are routinely used as monomers by which the essentially polymeric molecules are generated or produced, using a method that is essentially conform the sequential synthesis of polymers from monomeric molecules in nature. These single monomers are than spotted in a single fashion, one monomer thought to represent the full, or nearly the full binding site, without taking into consideration the multiple parts of a binding site constituting a discontinuous binding site.

The invention provides the recognition that essentially using dimeric or even larger (tri-, oligo-, or multimeric) segments in combination, thus in pairs or threesomes or

even more, offers distinct advantages. It not only provides a faster method to arrive at or recognise a molecule composed of various segments, it also provides for fast and efficient shuffling of segments to generate a molecule or test entity repertoire for the desired library. The invention for example provides a method wherein synthesis is started with a monomer in close proximity to which a second segment comprising a dimer, such as a dinucleotide or a dipeptide is spotted. Herein, a segment comprising a dimer at least consists of a dimer but can also be for example a trimer, or any other multimer, linking monomers of any nature, as required. Of course, once two segments have been spotted in close proximity, further segments can be added thereto.

In a preferred embodiment, to speed up further synthesis, or to be able to select distinct desired segments, the invention provides a method wherein said first segment also comprises a dimer, and in a yet even more preferred method, further segments comprise dimers as well. In a preferred embodiment, said dimer comprises a dinucleotide or dipeptide, but of course other dimers can be made also. The invention is further explained in the detailed description where several of the examples relate to libraries comprising molecules wherein each of said segments comprises a peptide, such as a tri-, a penta, an octa-, or nonapeptide; it is however also provided by the invention to use longer segments, e.g. 10-15, 15-20, 20-30 or 30-40 amino acids or nucleic acids long or longer and to use of a varied nature, e.g. wherein one comprises a nucleic acid and another comprises a peptide, to better mimic binding sites that are for example found on nucleic acid-protein complexes.

In a preferred embodiment, as for example shown in the examples, the invention provides a method wherein said first segment is spotted or attached to the solid phase by a thioether bond next to said second segment, however, the invention is of course not limited thereto. Nucleotide/side segments can for example be covalently linked or ligated by splicing enzymes or ligases, or by overlapping a first segment and the second segment with an in essence relatively short nucleotide strand that is partly complementary to both segments.

The invention thus provides a molecular library allowing testing for, identification, characterisation or detection of a continuous or discontinuous binding site capable of interacting with a binding molecule, said library having been provided with pluralities (pairs, threesomes, foursomes, fivesomes, sixsomes) of segments, each plurality preferably comprising at least one first segment spotted in close proximity

to a second segment, wherein at least said second segment previously existed as dimer or multimer. Preferably, each segment or part thereof having the capacity of being a potential single part of a discontinuous binding site, preferably wherein each of at least a first and a second segment or part thereof represents a potential single part of a discontinuous binding site. Such a library can for example exist of a synthetic molecular library made by chemical spotting of segments.

Preferably, such segments have distinct features, for example by being in essence segments that are, comprise or mimic molecular components of living organisms, such as (combinations of) nucleotides, sugars, lipids, amino acids, nucleic acid molecules (DNA or RNA), peptide nucleic acid molecules (PNA), carbohydrates, fatty acids or fats.

Herewith the invention provides synthesis of molecules comprising separate segments potentially representing at least two distinct parts of a discontinuous binding site, said parts not necessarily first being selected after previous identification of potential constituting parts, thereby allowing testing for discontinuous binding sites in a rapid and straight forward fashion.

The invention thus now allows identifying discontinuous binding sites of receptor molecules that interact or bind at a contact site with a hormone, a peptide, a drug, an antigen, an effector molecule or with another receptor molecule, of enzymes that bind with their substrate, of antibody molecules that bind with a binding site on an antigen, nucleic acid that binds with protein, and so on. In a preferred embodiment of the invention, at least one of said segments comprises a peptide, another segment being for example DNA, RNA, PNA, carbohydrate, a fatty acid, a peptide, an hormone or an organic molecule altogether. In one embodiment of the invention, all segments comprise a peptide. In this way a plurality of different binding bodies is synthesised by spotting a first segment next to a second, and so on, and on a second position in the test or library format yet another first segment is linked to a second, and so on, after which the synthesised binding bodies are each tested with the binding molecule in question, allowing the determination of a discontinuous antigenic determinant or discontinuous epitope of importance in for example a nucleic acid, a protein or peptide sequence.

Said peptide segment comprises at least 2 amino acids, and can in principle be as long as desired, e.g. containing a hundred amino acids or even more. In preferred

practice, said peptide segment comprises from 3 to 30, preferably from 4 to 20, even more preferably from 5 or 6 to 12 to 15 amino acids, such as 9 or 12 amino acids. Separate segments of course do not necessarily have to be of equal length.

Furthermore, peptide segments to be spotted together or at least in close proximity to each other can be selected at random, or under guidance of (a) known protein or peptide sequence(s). Selection at random provides a random library according to the invention. Selection from known protein or peptide sequences is for example useful when it is desired to find out whether a discontinuous binding site is composed of distinct sites or parts present at distinct proteins or peptides, for example in a protein complex to which a particular binding molecule can bind. Selection of various peptide segments from one known protein or peptide sequence is useful when it is desired to find out whether a discontinuous binding site is composed of distinct sites or parts present at one protein or peptide, for example at a folded protein to which a particular binding molecule can bind. Selection of peptide segments can be done by selecting overlapping peptides from such a known sequence. Overlapping peptides can have for example all but one or two amino acids in common, preferably overlapping in a contiguous fashion, or can overlap with only one or several amino acids. For a quick scan for discontinuous binding sites on a known protein, it is for example useful to select nonapeptide segments from said protein sequence, of which one has for example a 5-amino acid long overlap with another peptide segment. Equally useful, however, is to select tripeptide segments from said sequence having an overlap of only one amino acid, and use three, or even more segments in constructing the putative binding site molecule to which the to be tested binding molecule can bind.

Of course, such selection strategies are equally applicable to segments of a different nature, nucleic acid segments, comprising a certain number of nucleotides, such as 5, 7, 9, and so on, can be selected from known nucleic acid sequences comprising putative or sought after discontinuous binding sites, each segment selected from a certain position in said known nucleic acid sequence, if desired also in a overlapping fashion. Said nucleic acid segment comprises at least 2 nucleotides (be it DNA, RNA or PNA, or functional equivalents thereof), and can in principle be as long as desired, e.g. containing a hundred nucleotides or even more. In preferred practice, said nucleic segment comprises from 3 to 30, preferably from 4 to 20, even more preferably from 5

or 6 to 12 to 15 nucleotides, such as 9 or 12 nucleotides. Separate segments of course do not necessarily have to be of equal length, and, as said before, can even be of a different nature, e.g. peptide with DNA.

The segments can for example be chemically attached to the solid phase by chemical links or bonds. The links or bonds can be formed using many combinations of strategies of for example peptide or nucleotide chemistry and selective ligation reactions as known in the art. Ligation chemistry has been published, for instance, by groups of Kent (Ph.E.Dawson et al., Synthesis of Proteins by Native Chemical Ligation, Science 266 (1994) 776-779), Tam (J.P.Tam et al., Peptide Synthesis using Unprotected Peptides through Orthogonal Coupling Methods, Proc. Natl. Acad. Sci. USA 92 (1995) 12485-12489; C.F.Liu et al, Orthogonal Ligation of Unprotected Peptide Segments through Pseudoproline Formation for the Synthesis of HIV-1 Protease Analogs, J.Am.Chem.Soc. 118 (1996) 307-312; L.Zhang & J.P.Tam Thiazolidone Formation as a General and Site-specific Conjugation Method for Synthetic Peptides and Proteins, Analytical Biochemistry 233 (1996) 87-93), and Mutter (G.Tuchscherer & M.Mutter, Protein Design as a Challenge for Peptide Chemists, J.Peptide Science 1 (1995) 3-10; S.E.Cervigni et al, Template-assisted Protein Design: Chimeric TASP by Chemoselective Ligation, Peptides: Chemistry, Structure and Biology, P.T.P Kaumaya & R.S. Hodges eds, Mayflower (1996) 555-557).

Possible strategies for the formation of links as preferably provided by the invention are for example are:

1. Said link with of a segment or segments with a solid phase is formed using a homo- or hetero-bifunctional linking agent (S.S.Wong: Chemistry of Protein Conjugation and Cross-Linking, CRC Press Inc, Boca Raton, Florida USA 1991). In this construction a reactive group in a segment is used to react with one part of the bifunctional linking agent, thus facilitating the second part of the linking agent to react with a reactive group from a solid phase, or visa versa. For instance, a linker like MBS (m-maleinimidobenzoic acid N-hydroxysuccinimide ester) can be used to react via its active ester (succinimide) with an amino group of one segment and via its maleinimide group with a free thiol group from a solid phase, or visa versa. In this strategy, when linking preferably no other free amino- or thiol groups should be present in the segment. In order to accomplish this, the amino or thiol groups that

should be involved in the reaction can be deprotected selectively, for instance, by using a side chain protecting group that can be cleaved by a mild reagent like 1% trifluoroacetic acid, which leaves other side chain protecting groups intact.

2. Said link is formed by introduction of a modified amino acid in the synthesis of one or more segments. Amino acids can be modified, for instance, by introduction of a special group at the side-chain or at the alpha-amino group. A modification at the alpha-amino group leads to an amide or backbone modified peptide (see for example Gillon et al., Biopolymers, 31:745-750, 1991). For instance, this group can be a maleinimido group at the side chain amino group of lysine. At the end of the peptide synthesis this group will react fast and selective with a thiol group of a solid phase. Tam et al. (PNAS 92:12485-12489, 1995) described a synthesis of a peptide with a lysine residue that was modified in the side chain with a protected serine residue. After deprotection and selective oxidation using periodate, the alpha-amino, beta-hydroxy function of the serine is converted into an aldehyde function that can be ligated selectively with another thiol-bearing surface. Also peptide backbone links, via groups attached to the amide groups of the peptide, can be used to spot segments (Bitan et al., J. Chem. Soc. Perkin Trans.1:1501-1510, 1997; Bitan and Gilon, Tetrahedon, 51:10513-10522, 1995; Kaljuste and Unden, Int. J. Pept. Prot. Res. 43:505-511, 1994).

3. Yet another way to form said link is to synthesise a segment, such as a peptide, with a modified N-terminus. For instance, an N-terminal alpha-haloacetamido group can be introduced at the end of the synthesis. This group reacts fast and selectively with a solid phase which contains a thiol group. For instance, the first segment is synthesised with an N-terminal bromoacetamide and the solid phase is provided with a cysteine. Although most alpha-haloacetamide groups, like chloro-, bromo-, or iodoacetamide, will react with thiol groups, in those cases where speedy assembling is required, the bromoacetamide group is preferred because of its ease of introduction and fast and selective reaction with thiol groups.

Furthermore, the invention provides the possibility to address the link in every position of the first and/or the second or consecutive segment. For instance, for peptide segments sets of peptides are synthesised in which a cysteine or a side-chain modified lysine, both amino acid residues in a preferred embodiment being able to ligate selectively with another segment, shifts from the N-terminal amino acid

position one by one to the C-terminal amino acid position. Combinations of these possibilities will again lead to libraries as provided by the invention.

In another preferred embodiment, said segments are at least linked twice in close proximity to said solid phase, preferably by linking the respective ends of the segments to the surface, so that, so-to-speak, looped segments are attached to the solid phase. In such a preferred embodiment, pairs (or larger pluralities) of looped segments are attached to the solid phase, presenting themselves as binding bodies.

In a preferred embodiment, the invention provides a library wherein said pluralities are positionally or spatially addressable, e.g. in an array fashion, if desired aided by computer directed localisation and/or recognition of a specific pair or threesome (or larger plurality) or set of pluralities within the dimensions (e.g. plane or surface) of the support or solid phase of the library used. In an array, said pluralities are for example addressable by their positions in a grid or matrix.

A preferred embodiment of the invention further allows upscaling of the synthesis concerning the number of constructs on for example a solid support per cm^2. To facilitate generation of a great many possible constructs, containing for example test entities (pairs, threesomes or larger pluralities) comprising at least two peptide segments of a protein, many thousands of peptide constructs are made. For instance, when all constructs, in which both segments are for instance 12 amino acids long, are derived from a small protein with a length of 100 amino acid residues are needed, already 89 x 89 = 7921 peptide constructs are made, if the segments are only linked to the solid phase, for instance, via the C-terminus for the first segment and the N-terminus of the second segment, or visa versa, or both, using only one type of link. For a protein with a length of 1000 amino acid residues at least 989 x 989 = 978121 constructs are made. For efficient ELISA testing of these numbers of constructs, high construct densities on the solid support are preferred. High densities of constructs on a solid support are provided by the invention, wherein for instance, (a layer of) a first segment with a bromoacetamide group at the N-terminus is synthesised on a surface of, for instance, 1 cm^2. On yet another part of the surface, another first-segment may be applied. On each of such a peptide-functionalised surface of the support a set of, for instance, 10, preferably 50, preferably 100, or more second peptide segments containing a free thiol group are spotted or gridded, in a positionally or spatially addressable way, giving, after coupling, so many different peptide pairs. Spotting can,

for instance, be done using piezo drop-on-demand technology, or by using miniature solenoid valves. Gridding can, for instance, be done using a set of individual needles that pick up sub-microliter amounts of segment solution from a microtiter plate, containing solutions comprising the second segments. After the linking reaction, subsequent deprotection and extensive washing of the support to remove uncoupled peptide gives at least a peptide construct pair density as large as 10 to 50, or even 100 to 200, or up to 50 to 1000 spotted pairs per cm^2. This density allows to screen a great many possible peptide pairs or binding bodies derived from said proteins for binding with an antibody. For example: in a preferred embodiment 20000 to 100.000 constructs are made on 1000 cm^2, typically the surface is than screened for binding in ELISA with 100 ml of antibody solution, containing 1 - 10 µg of antibody/ml. For example, indirect or direct fluorescence detection allocates antibody binding constructs. Direct fluorescence detection with confocal scanning detection methods for example allows antibody detection on spots generated with droplets peptide-solution in the sub-nanoliter range, making even higher construct densities feasible. Of course, nucleic acid libraries can be made in a similar fashion.

Furthermore, the invention provides a solid support comprising a library according to the invention, said solid support allowing presentation of a potential discontinuous or conformational binding site or epitope to a binding molecule, said solid support having been provided with a plurality of test entities, each pair or threesome or larger plurality of said test entities or binding bodies being a possible representative of said binding site or epitope and for example comprising at least one first peptide or nucleotide for example covalently linked to a solid phase and a second peptide or nucleotide.

In a preferred embodiment, said solid support comprises at least a spot or dot (e.g. putative binding site, test entity, or pair of segments) density as large as 10, 20, or 50, or even 100, 200, or up to 500 or even 1000 spots per cm^2, preferably wherein said spots or dots are positionally or spatially addressable.

The invention further provides a method to screen for, i.e. test, identify, characterise or detect a discontinuous binding site capable of interacting with a binding molecule, comprising screening a library as provided by the invention with binding molecules, such as there are for instance antibodies, soluble receptors, which contain a Fc-tail or a tag for detection, receptors on cells, biotinylated molecules or fluorescent molecules.

Alternative segments could comprise, for instance, carbohydrates, non-natural amino acids, PNA's, DNA's, lipids, molecules containing peptide bond mimetics. In particular, the invention provides a method to screen for a discontinuous binding site capable of interacting with a binding molecule, comprising screening a library according to the invention with at least one teste entity and detecting binding between a member of said library and said test entity. In a preferred embodiment, said binding is detected immunologically, for example by ELISA techniques.

By detecting binding to a specific test entity (herein also called binding body) of said library, the invention said provides said member or binding body, a synthetic molecule comprising said binding body or test entity or pair or larger plurality of (looped) segments comprising a discontinuous binding site identifiable or identified or obtainable or obtained by a method according to the invention. Thus the invention provides use of a library according to the invention, use of a solid support or solid phase or array surface provided with one or more binding bodies or test entities according to the invention, or use of a method according to the invention for identifying or obtaining a synthetic molecule comprising a discontinuous binding site or a binding molecule capable of binding therewith. Because now discontinuous binding sites are provided, such a synthetic molecule can advantageously be used in vitro or in vivo for finding a binding molecule, and for effecting and/or affecting binding to a binding molecule, for example to interact or bind with receptor molecules that normally interact or bind with a hormone, a peptide, a drug, an antigen, with an effector molecule, with an agonist, with an antagonist, or with another receptor molecule; with enzymes that normally bind with their substrate; with antibody molecules, with nucleic acid, with protein, in short, with biomolecules. The invention is further explained in the detailed description without limiting the invention.

Figure legends.

Fig. 1.

6 different cysteines that can be used in coupling of bromine under different conditions .

Fig. 2 (spotting with dark colouring)

Analysis of two different peptides for showing the advantageous effect of two-sided linking and the formation of loops. On the left the peptide has a amino-terminal Br. On the right the peptide has a amino-terminal Br and a C-terminal Lysine-Br (synthesised as described in legend Fig. 4B).

Test was carried out in a miniwell setup (3ul each well). Surface is functionalised with thiolgroups (-SH groups). Peptides were coupled to the surface using the bromine (Br) - group of the peptide. Different concentrations of peptide were used for coupling to the surface. Two sets of peptides were used, one with one Br-group and the other (differs only from the previous peptide by an extra lysine + Br-acetyl moiety on the C-terminal site of the peptide) with two Br-groups. Binding was determined using differed antibody concentrations in an Elisa setup.

Fig. 3.

Proximity of segments after coupling on solid-support. On the left side: on a minimal distance of 2 angstrom linkers of 15 angstrom are coupled. The segments are coupled to these linkers. The flexibility of the linkers allows that the termini of the two segments move within distances of 0-30 angstrom. On the right side: the distances between the linkers can be varied from 2 to 50 or more. As an example 9 angstrom is shown. This allows that the termini of the two segments move within distances of 0-40 angstrom.

Fig. 3B.

Schematical representation of how the two segments are linked as loops to the poly-carbon polymer surface. The preferred distances, at least in the case of CDR derived binding bodies, between de top of the loops are 0-30 angstrom which is similar to that of the CDR's in an antibody.

Fig. 4.

Schematical representation of how two segments can be coupled onto the (polycarbon)-polymer surface. The graph shows four examples. In example-1 two linear segments are coupled. In example-2 two looped segments are coupled. In example-3 two segments are coupled as loops. In example-4 two segments are coupled as loops. With extra spaced building blocks (for example phenylalanine amino acids) two obtain extended loops. On the (polycarbon)-surface two types of protected cysteines (cys (trt) and cys (mmt)) and for example one spacing building block is coupled. The cys (mmt) is deprotected with 1% TFA while the cys (trt) remains protected. The first segment is coupled to the deprotected cys (mmt). Then the second cys (trt) is deprotected with 95% TFA. Then the second segment is coupled to the now deprotected cys (trt).

Fig. 4B.

Schematical representation of how two segments can be coupled onto a cyclic template that itself is coupled to the polymer surface. The cyclic template is a cyclic flexible peptide. The cyclic peptide contains four lysines (mmt), two cysteines (trt) and two cysteines (butyl). The peptide is coupled to the resin via a sulphur that is sensitive to 1% TFA. At the amino-terminus a bromine is attached as described previously. The procedure is as follows: The synthesized peptide is treated with 1% TFA. This results in deprotection of the lysines and de-coupling of the peptide from the resin. The cysteines remain protected. After raising the pH to 8 the N- and C-terminus of the peptide are linked through the S and Br. Then the –NH2 on the deprotected lysines is coupled to Br. The resulting cyclic peptide, with four Br and still four protected cysteines, is coupled to the linkers via the Br. To the cyclic template coupled to the linker-cysteines two peptide segments are coupled. First the two cysteines (trt) are deprotected with 95% TFA. Then, the first segment is coupled.

Second the two cys (butyl) are deprotected with NaBH4. Then, the second segment is coupled.

Fig. 4C.

Schematical representation of how two segments can be coupled onto two other segments that are coupled to the polymer surface. With free –SH on the surface two segments are coupled to the surface via a N- and C-terminal Br. The N-terminal Br is synthesized as described previously. The C-terminal Br is linked to a C-terminal Lysine as described in Fig. 4B. Both segments contain protected cysteines on which two other segments are coupled also as described in Fig. 4B.

Fig. 5.

Schematical representation of matrix-scan with two segments. On the polymer surface a mixture of cys (mmt) and cys (trt) are coupled. After 1 % TFA the cys (mmt) is deprotected. Then, in each square one peptide is coupled via one or two terminal Br. Thus, peptide-1 in square –1, peptide-2 in square-2 etc. to peptide-100 in square-100. Then the cys(trt) is deprotected with 95% TFA. Then , in each square 100 different peptides are spotted. Thus, peptide-1 to 100 in square-1, peptide 1-100 in square-2 etc. to peptide-1 to peptide-100 in square-100.

Fig. 6. Binding-assay of all overlapping 30-mers covering the linear sequence of hFSHR with the biotinylated synthetic 40-mer hFSH-peptide biotin-EKEEARFCISINTTWAAGYAYTRDLVYKDPARPKIQKTAT-CONH2. The 30-mer peptides were spotted as described and the 40-mer peptides were synthesized using standard FMOC-chemistry. The various 30-mer peptides were incubated with 1 microgram/ml hFSH-peptide. After washing the peptides were incubated with streptavidin-peroxidase, and subsequently after washing, with peroxidase substrate and H2O2.

Fig.7. Schematic representation of the development of synthetic mimics of discontinuous binding sites on the hTSHR and hTSH. On thyroid cells the hTSH-receptor binds hTSH. The autoimmune antibodies from Graves and Hashimoto patients also bind the hTSH-receptor. Through screening of all overlapping 30-mers

of hTSH segments of the discontinuous binding site for hTSHR are identified (as described for FSH, see legend Fig. 6). Through screening of all overlapping 30-mers of hTSHR segments of the discontinuous binding sites for Graves and Hashimoto antibodies are identified. Through modeling and usage of synthetic templates the individual segments are combined into one discontinuous synthetic mimic.

Fig.8. Schematic representation of an array comprising synthetic mimics of discontinuous binding sites or binding bodies. Binding bodies are selected and improved by making arrays that contain a multiplicity of spatially addressable binding bodies on the solid surface (or alternatively, on a separate molecular scaffold). The arrays can be incubated with target to screen for binding bodies that bind the target of interest. Lead binding bodies can be improved by making follow-up arrays composed of multiple variants of the lead binding bodies, e.g. by sequence shuffling. If the desired specificity and/or affinity is reached, the binding bodies can be produced onto a scaffold and produced and used in bulk.

Fig.9. Schematic representation of the development of synthetic mimics of discontinuous binding sites or binding bodies derived from CDR sequences. Binding bodies are constructed by positioning on a solid pahse or array surface (preferably a (polycarbon)-polymer surface) or on predefined scaffolds or templates. Binding bodies can be derived from the Complementarity Determining Regions (CDR's) of antibodies or any other protein motif that is known to bind other molecules, preferably with high affinity.

Fig. 10

Standard linear pepscan on all overlapping synthetic 12-mers covering the linear sequence of hTNF with monoclonal antibody 210 (R&D Systems, MAB210, clone 1825.12, through ITK Diagnostics Uithoorn, The Netherlands). A small peak with the sequence IKSPCQRETPEG was identified. The y-axis are optical density values (OD) obtained using a ccd-camera system. Rampo, rabbit-anti-mouse peroxidase (DAKO).

Fig. 11

Partial listing of peptides synthesized for loop-loop 15-mer Matrix-scan. All overlapping 15-mer loop-peptides covering the linear sequence of human tumor necrosis factor (hTNF) were synthesized, i.e. 145 hTNF loop-peptides in total. Z is a Cys-butyl. The amino terminus of all peptides contain a bromo-group (+).

Fig. 12

Configuration of the loop-loop 15-mer Matrix-scan. Schematical representation of matrix-scan with two loop segments. On the polymer surface a mixture of cys (mmt) and cys (trt) are coupled. After 1 % TFA the cys (mmt) is deprotected. Then, in each square one peptide is coupled via its N-terminal Bromo-group (+). Thus, peptide-1 in square –1, peptide-2 in squeare-2 etc. until peptide-145 in square-145. Then the cys(trt) is deprotected with 95% TFA. Then , in each square 145 different peptides are spotted simultaneously. Thus, peptide-1 to 145 in square-1, peptide 1-145 in square-2 etc. to peptide-1 to peptide-145 in square-145. Some extra squares were used for controls (linear epitopes).

Fig. 13

Result of the loop-loop 15-mer Matrix-scan with anti-hTNF mAb 210 (10 ug/ml). The result obtained with all 145 squares are plotted. Squares 66, 67 and 92-96 are clearly labelled (firstly coupled loop-peptides). On top of these and other squares spots are labelled as well (spots represent first peptide coupled next to second loop peptide). Identified squares en spots: Sq-65: +FKGQGCPSTHVLLTZ; Sq-66: +KGQGCPSTHVLLTHZ; Sq-67: +GQGCPSTHVLLTHTZ; Sq-87: +SYQTKVNLLSAIKSZ; Sq-88: +YQTKVNLLSAIKSPZ; Sq-94: +LLSAIKSPCQRETPZ; Sq-95: +LSAIKSPCQRETPEZ; Sq-127: +LEKGDRLSAEINRPZ; Sq-128: +EKGDRLSAEINRPDZ. Peptide-65: +FKGQGCPSTHVLLTZ; Peptide-70: +CPSTHVLLTHTISRZ; Peptide-72: +STHVLLTHTISRIAZ ; Peptide-77: +LTHTISRIAVSYQTZ; Peptide-94: +LLSAIKSPCQRETPZ; Peptide-95: +LSAIKSPCQRETPEZ; Peptide-99:

+KSPCQRETPEGAEAZ; Peptide-126: +QLEKGDRLSAEINRZ; Peptide-129: +KGDRLSAEINRPDYZ. The y-axis is in arbitrary units.

Fig. 14

Result of the loop-loop 15-mer Matrix-scan with mAb 210 (10 ug/ml) with details of squares 65 and 127. Combination of loop-peptide 65 with loop-peptides 94,95, combinations of loop-peptide 65 with 126-127, combinations of loop-peptide 127 with loop-peptides 65-77 and combinations of loop-peptide 127 with loop-peptides 94-96 are labelled. The y-axis is in arbitrary units.

Fig. 15

Three dimensional representation of the identified binding loop-loop peptides with mAb-210 (10 ug/ml). Shown are the three regions identified (peptides 65-69, 94-96 and 126-127): GQGCPSTHVLLTHTIS (VLLT are labeled); SAIKSPCQRE (KSPC are labeled); KGDRLSAEINR (SA are labeled).

Fig. 16

Result of the loop-loop 15-mer Matrix-scan of loop-loop CDR-regions of antibodies with lysozyme-biotin (100 ug/ml, in triplo). Using 1 ug/ml lysozyme-biotin no binding is observed (not shown). Controls of only streptavidin-peroxidase in between the tests were negative (not shown).

Peptides A, B, C, D, E and F: Peptide-A: +ARERDYRLDYZ (HCDR3 of 1fdl.pdb); Peptide-B: +ARGDGNYGYZ (HCDR3 of 1mlb.pdb); Peptide-C: +LHGNYDFDGZ (HCDR3 of 3hfl.pdb); Peptide-D: +ANWDGDYZ (HCDR3 of 3hfm.pdb); Peptide-E: +ARRYGNSFDYZ (HCDR3 of 1qfw.pdb); Peptide-F: +ARQGTAAQPYWYZ (HCDR3 of 1qfw.pdb) (1 fdl.pdb, 1 mlb.pdb, 3hfl.pdb and 3hfm.pdb are antibodies that bind lysozyme; 1 qfw.pdb are two antibodies that bind human choriogonadotrophin). All peptides have an amioterminal bromo-group (+) and a carboxyterminal lysine-mmt (Z).

Peptides 1 to 27: Peptide-1: +RASGNIHNYLAZ (LCDR1 of 1fdl.pdb); Peptide-2: +RASQSISNNLHZ (LCDR1 of 1mlb.pdb); Peptide-3: +SASSSVNYMYZ (LCDR1 of 3hfl.pdb); Peptide-4: +RASQSIGNNLHZ (LCDR1 of 3hfm.pdb); Peptide-5: +RASESVDSYGNSZ (LCDR1 of 1qfw.pdb); Peptide-6: +ASESVDSYGNSFZ (LCDR1 of 1qfw.pdb); Peptide-71: +SESVDSYGNSFMZ (LCDR1 of 1qfw.pdb); Peptide-8: +ESVDSYGNSFMQZ (LCDR1 of 1qfw.pdb); Peptide-9: +RASESVDSYGNSFZ (LCDR1 of 1qfw.pdb); Peptide-10: +ASESVDSYGNSFMZ (LCDR1 of 1qfw.pdb); Peptide-11: +SESVDSYGNSFMQZ (LCDR1 of 1qfw.pdb); Peptide-12: +RASESVDSYGNSFMZ (LCDR1 of 1qfw.pdb); Peptide-13: +ASESVDSYGNSFMQZ (LCDR1 of 1qfw.pdb); Peptide-14: +RASESVDSYGNSFMQZ (LCDR1 of 1qfw.pdb); Peptide-15: +KASETVDSFVSZ (LCDR1 of 1qfw.pdb); Peptide-16: +LLVYYTTTLADGZ (LCDR2 of 1fdl.pdb); Peptide-17: +LLIKYVSQSSSGZ (LCDR2 of 1mlb.pdb); Peptide-18: +RWIYDTSKLASGZ (LCDR2 of 3hfl.pdb); Peptide-19: +LLIKYASQSISGZ (LCDR2 of 3hfm.pdb); Peptide-20: +LLIYRASNLESGZ (LCDR2 of 1qfw.pdb); Peptide-21: LLIFGASNRESGZ (LCDR2 of 1qfw.pdb); Peptide-22: +QHFWSTPRTZ (LCDR3 of 1fdl.pdb); Peptide-23: +QQSNSWPRTZ (LCDR3 of 1mlb.pdb); Peptide-24: +QQWGRNPTZ (LCDR3 of 3hfl.pdb); Peptide-25: +QQSNSWPYTZ (LCDR3 of 3hfm.pdb); Peptide-26: +QQSDEYPYMYTZ (LCDR3 of 1qfw.pdb); Peptide-27: +GQTYNHPYTZ (LCDR3 of 1qfw.pdb) (1 fdl.pdb, 1 mlb.pdb, 3hfl.pdb and 3hfm.pdb are antibodies that bind lysozyme; 1 qfw.pdb are two antibodies that bind human choriogonadotrophin). All peptides have an amioterminal bromo-group (+) and a carboxyterminal lysine-mmt (Z).

The loop-loop peptide pair, +LHGNYDFDGZ +SESVDSYGNSFMQZ (loop of HCDR3 of 3hfl.pdb with loop of LCDR1 of 1qfw.pdb) that has the highest binding activity is indicated by arrow.

Fig. 17

Result of pepscan elisa with two different antibodies on single or double peptide loops coupled to pepscan minicards as described above. Coupled to square-A: Loop peptide-1; Coupled to square-B: first Loop peptide-1 followed by Loop peptide-2; Coupled to square-C: Loop peptide-2; Coupled to square-D: first Loop peptide-2 followed by Loop peptide-1. Loop peptide-1: +KSYNRVTVMGGFKVEZ-conh2; Loop peptide-2:

+LQENPFFSQPGAPILZ-conh2. The y-axis are optical density values (OD) obtained using a ccd-camera system. Both loop-peptides are derived from human Follicle-Stimulating Hormone (hFSH).

Detailed description

SYNTHESIS OF PEPTIDE CONSTRUCTS

A polypropylene or polyethylene support, or of other suitable material, was grafted with, for instance, polyacrylic acid. As an example: a polypropylene support in a 6 % acrylic acid solution in water, containing $CuSO_4$, was irradiated using gamma radiation at a dose of 12 kGy. The grafted solid support containing carboxylic acid groups was functionalised with amino groups via coupling of t-butyloxycarbonyl-hexamethylenediamine (Boc-HMDA) using dicyclohexylcarbodiimide (DCC) with N-hydroxybenzotriazole (HOBt) and subsequent cleavage of the Boc groups using trifluoroacetic acid. Subsequently the surface is functionalised with (when preferred a mixture of differently protected) Cys amino acids using standard Fmoc chemistry. Examples of differently protected Cys groups are Cys (Trt) and Cys (mmt). After removal of the FMOC the amino group is acetylated. Side chain deprotection can be done as described. Standard Fmoc peptide synthesis chemistry was used to link peptides (segments) on to the amino functionalised solid support. After cleavage of the Fmoc group of the last amino acid and washing, bromoacetic acid was coupled using DCC or DCC/HOBt. A second bromoacetic acid (in the same step) can be coupled to the surface when for example a lysine (Lys) residue is present in the peptide: The side chain protection chemistry of Lys (using FMOC-Lys(MTT)-OH) allows that only the amino group of the Lys-side chain is liberated (with 1% trifluoracetic acid in dichloromethane)while the other amino acids still stay protected. Subsequently, if only DCC was used the peptide did contain a thiol reactive bromoacetamide group, however, if DCC/HOBt was used to couple bromoacetic acid, the peptide essentially did not contain the bromo group, but another reactive group capable to react efficiently with thiol groups thus forming the same thioether link between the segments. Coupling/ligation of a second peptide next to a peptide coupled or synthesised on a solid support: Bromo functionalized peptides can be coupled to the solid support (when a thiol is present) in an aquous solution containing a sodiumbicarbonate buffer at about ph 7-8. Peptides were synthesised at polyethylene pins grafted with poly-hydromethylmethacrylate (poly-HEMA). This

graft polymer was made by gamma irradiation of polyethylene pins in a 20% HEMA solution in methanol/water 80/20 or 70/30 at a dose of 30-50 kGy. The functionalised support can be used for the synthesis of 1 μmol of peptide/cm^2 after coupling of β-alanine and an acid labile Fmoc-2,4-dimethoxy-4'-(carboxymethyloxy)-benzhydrylamine (Rink) linker. The peptides were synthesised using standard Fmoc chemistry and the peptide was deprotected and cleaved from the resin using trifluoroacetic acid with scavengers. The cleaved peptide containing a cysteine residue at a concentration of about 1 mg/ml was reacted with the solid support described above in a water/sodium bicarbonate buffer at about pH 7-8, thus forming a partially protected construct of two peptides each at least once covalently bound via a thioether bond to the solid support. The construct described above was deprotected following standard procedures using trifluoroacetic acid/scavenger combinations. The deprotected constructs on the solid support were extensively washed using disrupting buffers, containing sodium dodecylsulphate and β-mercaptoethanol, and ultrasonic cleaning and were used directly in ELISA. Subsequent cleaning in the disrupt buffers allows repeatingly testing against antibodies in ELISA.

According to these methods a library of constructs, for example consisting of a dodecapeptide segment coupled via its C-terminally added cysteine residue next to a N-terminally bromoacetylated second segment, allowing scanning a protein sequence for example by steps of a single amino acid residue. The bromoacetamide peptide was covalently bound to a functionalised polypropylene/polyacrylic acid solid support in 3 μl wells as described above. The cysteine-containing sequences are synthesised on and cleaved from functionalised polyethylene pins as described above. On a surface of a solid support peptides are synthesized as described above. On this peptide functionalized support a second peptide segment containing a free thiol group was spotted using piezo drop-on-demand technology, using a microdosing apparatus and piezo autopipette (Auto Drop-Micropipette AD-K-501) (Microdrop Gesellschaft fur Mikrodosier Systeme GmbH. Alternatively, spotting or gridding was done using miniature solenoid valves (INKX 0502600A; the Ice Company) or hardened precision ground gridding pins (Genomic Solutions, diameters 0.4, 0.6, 0.8 or 1.5 mm). Subsequent deprotection of the construct and extensive washing to remove uncoupled

peptide gave binding body constructs at the spotted area. Peptide constructs generated with peptide solution droplets in the nanoliter-range, bind enough antibody for detection, in this case using indirect fluorescence detection. Spots generated with 0.25 nl - 50 nl are smaller than 1 mm^2. Thus, in this set-up binding body density can be as large as 100-1000 spots per cm^2, when using smaller equipment, densities can even be higher.

In short, a thiol fuction is introduced on an amino-functionalised solid support. This can be made by a direct reaction of the amino groups with, for instance, iminothiolane, or by coupling of Fmoc-Cys(Trt)-OH, followed by Fmoc cleavage using piperidine, acetylation, and trityl deprotection using TFA/scavenger mixtures. This thiol-functionalised solid support can be reacted with, for instance, a bromoacetamide-peptide, containing a protected cysteine residue. After coupling of the first peptide, the cysteine can be deprotected, using, for instance, a TFA/scavenger mixture. As yet unused free thiol groups can be used to couple a second bromoacetamide-peptide, again containing a protected cysteine. This procedure can be repeated to make segment constructs. Several types of scans can be used in combination with this multi-segment scan.

Examples of use

Proteins and peptides can be screened using any type of binding molecule, e.g. biomolecules such as antibodies, soluble receptors, which contain a Fc-tail or a tag for detection, biotinylated molecules or fluorescent molecules. Alternative segments could be, for instance, carbohydrates, non-natural amino acids, PNA's, DNA's, lipids, molecules containing peptide bond mimetics.

TSH example

The design and synthesis of synthetic mimics of discontinuous binding sites of large proteins such as TSH or TSHR is currently desired. Toward this aim template based mimics of proteins have provided a powerful new tool for basic research. Technology hereien provided enables us to map discontinuous binding sites, couple these onto a synthetic template and monitor in detail the structural and functional characteristics.

Pivotal to this approach is the possibility of synthesising and testing of 100.000's of synthetic peptides in array-format. This is possible with the technologies provided herein. These include peptide-array synthesis and new methodology in template chemistry. Through chemistry all kinds of synthetic groups are coupled on two or more different positions on these templates, allowing reconstruction of the discontinuous binding sites and the synthesis of mimics. The development of methods that allows mapping of discontinuous binding sites between large proteins is a major research target. Various strategies have been adopted with moderate success. The most successful techniques to date include X-ray crystallography, Combinatorial libraries en Mass-Spectrometry. We provide a new approach involving peptide-arrays. Peptide array technology has long been used to identify short linear peptides involved in binding. All overlapping linear peptides (12-15-mers) of a given protein are synthesised on a solid-support such as plastic or paper and incubated with the target protein, most often an antibody. Those peptides that are recognised are so-called linear epitopes. Discontinuous epitopes could not be detected. Nevertheless, the early peptide-array technology laid the foundation for methods that identify discontinuous epitopes in a systematic fashion. This made it possible to couple on an array surface any part of a protein (for instance a peptide of 15 amino acids long) next to any other part of a protein (for instance a peptide of 15 amino acids long) in any orientation. These arrays with all possible combinations of peptides showed in our hands to allow accurate definition of discontinuous epitopes (Fig. 2). We now focus on discontinuous epitopes involved in Graves disease and Hashimoto disease, but others are as well within reach. The thyroid diseases are autoimmune diseases against the thyroid. The antibodies bind discontinuous epitopes on the thyrotropin receptor on the thyroid gland. Overactivation (Graves) or blockage (Hashimoto) of the thyroid gland leads to serious health problems. Mapping of both the antibody binding regions as well as the TSH binding region greatly contributes to the understanding of both diseases. Subsequently, hTSH- and hTSHR mimics of these discontinuous epitopes will be used in new diagnostic tools allowing early discovery of Graves and Hashimoto disease. Studies on human Follicle-Stimulating Hormone (hFSH) and its receptor (hFSHR) have revealed discontinuous binding sites. Biotinylated 40-mers covering various regions of hFSH were tested in a peptide-array binding-assay as herein provided on all overlapping 30-mers covering the linear sequence of hFSHR.

One of the 40-mers clearly bound to a receptor region (Fig. 1). Based on these results a similar study on the hTSH/hSHR couple hTSHR, a hormone-receptor couple that is structurally very similar to the hFSH/hFSHR couple, provides peptides that can be used as diagnostic tools for Graves and/or Hashimoto disease. Patients with Graves or Hashimoto disease develop antibodies against their own thyroid receptors which leads to hyper- or hypothyroidism, respectively. Although the population of antithyrotropin receptor antibodies are heterogeneous most Graves antibodies bind the N-terminus of the receptor whereas most Hashimoto antibodies bind the C-terminus of the receptor. In our study panels of Graves and Hashimoto sera are tested a) for binding in a peptide-array to the set of overlapping 30-mers covering the hTSH-receptor; b) in a competition-assay in which the binding of biotinylated 40-mer TSH-peptides to hTSH-receptor is competed with Graves and Hashimoto sera. In this way discontinuous binding sites are mapped. After mapping the discontinuous binding sites synthetic mimics are designed and synthesized. A primary strategy for synthesis of this kind of synthetic mimics is the synthesis of templates onto which the discontinuous epitope can be reconstructed. The use of templates facilitates the possibility to add various parts of the discontinuous epitope. In this way hardly any specific binding information will be lost by a high flexibility of the peptide constructs. Attachment of peptides to template structures will closely mimic the native discontinuous epitopes. Recently much progress has been made in this field. By using stable templates as a framework on which to couple recognition fragments, peptides can be obtained with desired activity.

Further examples

Examples of use:

Mapping discontinuous epitope on human Tumor Necrosis Factor (hTNF) (Figs. 10-15).

The monoclonal antibody mAb-210 raised against hTNF was tested on linear and loop peptides (mAb-210 was bought from R&D Systems, MAB210, clone 1825.12, through

ITK Diagnostics Uithoorn, The Netherlands). Firstly, it was tested in pepscan on all overlapping linear 12-mers covering hTNF. This resulted in a minor peak around sequence IKSPCQRETPEG (Fig. 10). Secondly, it was tested in pepscan matrix-scan on double 15-mer loop-loop peptides (as described in Figs 3 and 4 and explained through Figs 11-12). Two loop-regions were labelled: peptide sequence GQGCPSTHVLLT (squares 65 to 67) and SAIKSPCQRE (squares 92 to 96) (Fig. 13, 14). In addition in various squares loop peptide spots were identified corresponding to sequence GQGCPSTHVLLT(spots 65-67); SAIKSPCQRE (spots 92-96) and KGDRLSAEINR (spots 126-129) (Fig. 14). These three regions, illustrated in Fig. 15 on the three-dimensional model of hTNF, are located on one side of the hTNF molecule and form one large discontinuous epitope region.

Identification of synthetic mimics of antibodies (binding bodies) (Fig. 16).

From six different antibodies the HCDR3-region (complementary determining region three of the antibody heavy chain) was synthesized as synthetic loop-peptides. As an example four different anti-lysozyme antibodies and two different anti-choriogonadotrophin antibodies were selected 1fdl.pdb (D1.3), 1 mlb.pdb (D44.1), 3hfl.pdb (HyHel-5), 3hfm.pdb (HyHel-10) all anti-lysozyme, and 1qfw.pdb, two anti-human choriogonadotrophin, one anti-alpha and one anti-beta. The synthetic loop peptides were coupled to the minicards as described above. The three-dimensional coordinates (pdb-files) were extracted from the Protein Data Bank (PDB) at www.rcsb.org (RCSB, Research Collaboratory for Structural Bioinformatics) (Berman et al., 2000, The Protein Data Bank. Nucleic Acids Research, 28 pp. 235-242; Bernstein et al. 1977, The protein data bank: A computer-based archival file for macromolecular structures. J. Mol. Biol. 112 :535-542).

Together with each of the six peptides 27 different other loop peptides were coupled to the minicard as described in figure 3B: thus group-1 was a loop of HCDR3 of 1fdl.pdb coupled next to 27 different loops covering LCDR1, LCDR2 or LCDR3, group-2 was a loop of 1mlb.pdb coupled next to 27 different loops covering LCDR1, LCDR2 or LCDR3 etc. etc. (LCDR, complementary determining region three of the antibody light chain). The 27 different loop peptides represented LCDR1, LCD2 or LCDR3 of

the same antibodies described above (1 fdl.pdb, 1 mlb.pdb, 3hfl.pdb, 3hfm.pdb or 1qfw.pdb).

The result is shown in Fig. 16 (6 groups with 27 loop-loop coupled peptides). The six loop-loop coupled peptides with the highest binding activity were:+LHGNYDFDGZ +SESVDSYGNSFMQZ (loop of HCDR3 of 3hfl.pdb and loop of LCDR1 1qfw.pdb, respectively) (see Fig. 16); +LHGNYDFDGZ +RASESVDSYGNSFMQZ (loop of HCDR3 of 3hfl.pdb and loop of LCDR1 1qfw.pdb, respectively); +LHGNYDFDGZ +RASESVDSYGNSFZ (loop of HCDR3 of 3hfl.pdb and loop of LCDR1 1qfw.pdb, respectively); +LHGNYDFDGZ +ASESVDSYGNSFMZ (loop of HCDR3 of 3hfl.pdb and loop of LCDR1 1qfw.pdb, respectively); +LHGNYDFDGZ +ASESVDSYGNSFZ (loop of HCDR3 of 3hfl.pdb and loop of LCDR1 1qfw.pdb, respectively); +LHGNYDFDGZ +LLVYYTTTLADGZ (loop of HCDR3 of 3hfl.pdb and loop of LCDR2 1fdl.pdb, respectively).

The loop-loop peptide pair, +LHGNYDFDGZ +SESVDSYGNSFMQZ (loop of HCDR3 of 3hfl.pdb with loop of LCDR1 of 1qfw.pdb, respectively) that has the highest binding activity is indicated by an arrow (Fig. 16). This loop-loop peptide pair is derived from an anti-lysozyme antibody and an anti-human choriogonadotrophin antibody. The results shown in Fig. 16 shows that particular pairs of synthetic CDR's show better binding to lysozyme than other pairs, especially group-C. Therefore, loop-loop combinations of synthetic loops representing different CDR's of (different) antibodies, not necessarily derived from the original antibody which in this example is an anti-lysozyme antibody, can be used to identify lead synthetic compounds that mimic antibodies.

Construction of a double-loop mimic of an discontinuous epitope (Fig. 17). Two peptides that constitute two separate parts of a discontinuous epitope were coupled to the surface of a minicard as described above in legend of Fig. 12 (cf. Fig. 3A and Fig. 4 (example-4)). A cys(mmt) was coupled alone or in combination with a cys(trt) (in a 1:1 ratio) and/or val(mmt) (the cys and val in a 1:1, 1:3, 1:9 etc. ratio). In this way one peptide was coupled (squares A and C) or two peptides with increasing valines in between the cysteines were coupled (squares B and D) (cf. Fig. 4B

(example-4), Fig. 17). These four configurations were incubated with two different antibodies.

Antibody-1 recognized, when the individual loop peptides are coupled as a single loops, only loop peptide-2. Antibody-2 recognized, when the individual loop peptides are coupled as a single loops only loop peptide-1. When the two loop peptides are combined antibody-1 showed a higher binding activity with peptide-1 as coupled first. When the two loop peptides are combined antibody-2 showed not a higher binding activity.

The results shown in Fig. 17 shows that particular pair of synthetic loops of a discontinuous epitope show improved binding to a particular antibody. Therefore, combinations of synthetic loops that are part of a discontinuous epitope can be used to identify lead synthetic compounds that mimic discontinuous epitopes.

CLAIMS

1. A method for producing a molecular library for identification or detection of a binding site comprising providing said library with a plurality of test entities, further comprising generating at least one of said entities by spotting at a solid phase at least a first segment in close proximity to a second segment.

2. A method according to claim 1 wherein said solid phase comprises an array surface.

3. A method according to claim 1 or 2 wherein at least one of said segments comprises a peptide.

4. A method according to anyone of claims 1 to 3 wherein each of said segments comprise a peptide.

5. A method according to anyone of claims 1 to 4 wherein at least said first segment is linked by a thioether bond to said solid phase.

6. A method according to anyone of claims 1 to 5 wherein each of at least a first and/or a second segment or part thereof represents a potential part of a discontinuous binding site.

7. A library comprising a plurality of test entities comprising at least a first and a second segment obtainable by a method according to anyone of claims 1 to 6.

8. A library according to claim 7 wherein said test entities are positionally or spatially addressable.

9. A library according to claim 7 or 8 wherein each of at least a first and/or a second segment or part thereof represents a potential part of a discontinuous binding site.

10. A solid support comprising a library according to anyone of claims 7 to 9.

11. A method to screen for a binding site capable of interacting with a binding molecule, comprising screening a library according to anyone of claims 7 to 9 with at least one potential binding molecule and detecting binding between a test entity of said library and said potential binding molecule.

12. A method according to claim 11 wherein said binding site is a discontinuous binding site.

13. A synthetic molecule comprising a binding site identifiable or obtainable by a method according to claim 11 or 12.

14. A binding molecule comprising a binding site identifiable or obtainable by a method according to claim 11 or 12.

15. A molecule according to claim 13 or 14 wherein said binding site comprises a discontinuous binding site.

16. Use of a library according to anyone of claims 7 to 9, a solid support according to claim 10, or a method according to claim 11 or 12 for identifying or obtaining a synthetic molecule comprising a binding site.

17. Use of a library according to anyone of claims 7 to 9, a solid support according to claim 10, or a method according to claim 11 or 12 for identifying or obtaining a binding molecule capable of binding to a binding site.

18. Use of a molecule according to claim 13, 14 or 15 for interfering with or effecting binding to a binding molecule.

Cysteine protection.

Protecting Group	Cleavage Reagent	Comments
Acm	Hg^{2+} , I_2 , Ag^+ , Tl^{3+}	Stable to TFA. Enables peptide to be purified in a protected form prior to liberation of the easily oxidizable thiol groups. Removal of Acm and simultaneous disulphide bond formation can be carried out by treatment with I_2 or Tl^{3+}.
tBu	Hg^{2+} , TCIMS/ PhSoOh, TFMSA	Stable to TFA and iodine oxidation. Treatment with $MeSiCl_3$/ PhSOPh removes t-Bu and cyclizes in one step without scrambling existing disulphide bonds.
Trt	TFA, I_2 , Tl^{3+}	Most useful derivative for routine use in Fmoc SPPS as it generates the sulfydryl peptide directly from the TFA cleavage reaction.
tButhio	RSH, R_3P	Stable to TFA providing thiol scavengers are not used. Has been used in combination with Acm for selective formation of two disulphide bonds.
Mmt	1% TFA in DCM	Can be selectively removed whilst the peptide remains attaches to the solid phase. Ideal for on-resin disulphide bond formation or modification of Cys side-chain.
Npys	RSH, R_3P	Stable to TFA providing thiol scavengers are not used. Activates thiol groups towards disulphide bond formation. Useful for the selective preparation of mixed disulphides.

Fig. 1

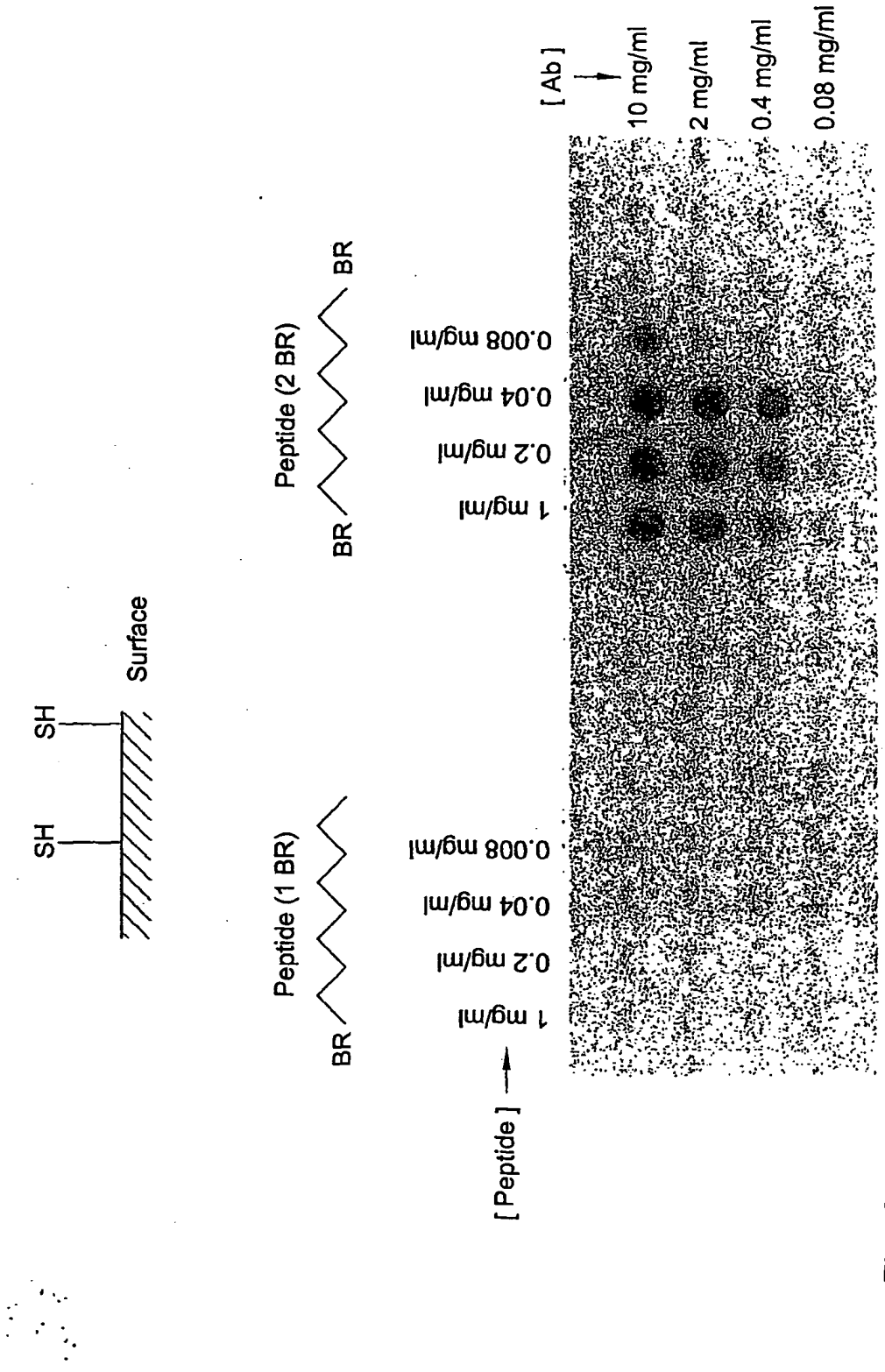

[Ab]
10 mg/ml
2 mg/ml
0.4 mg/ml
0.08 mg/ml
[Peptide]
1 mg/ml
0.2 mg/ml
0.04 mg/ml
0.008 mg/ml
Peptide (1 BR)
BR
Peptide (2 BR)
BR
BR
SH
SH
Surface

Fig. 2

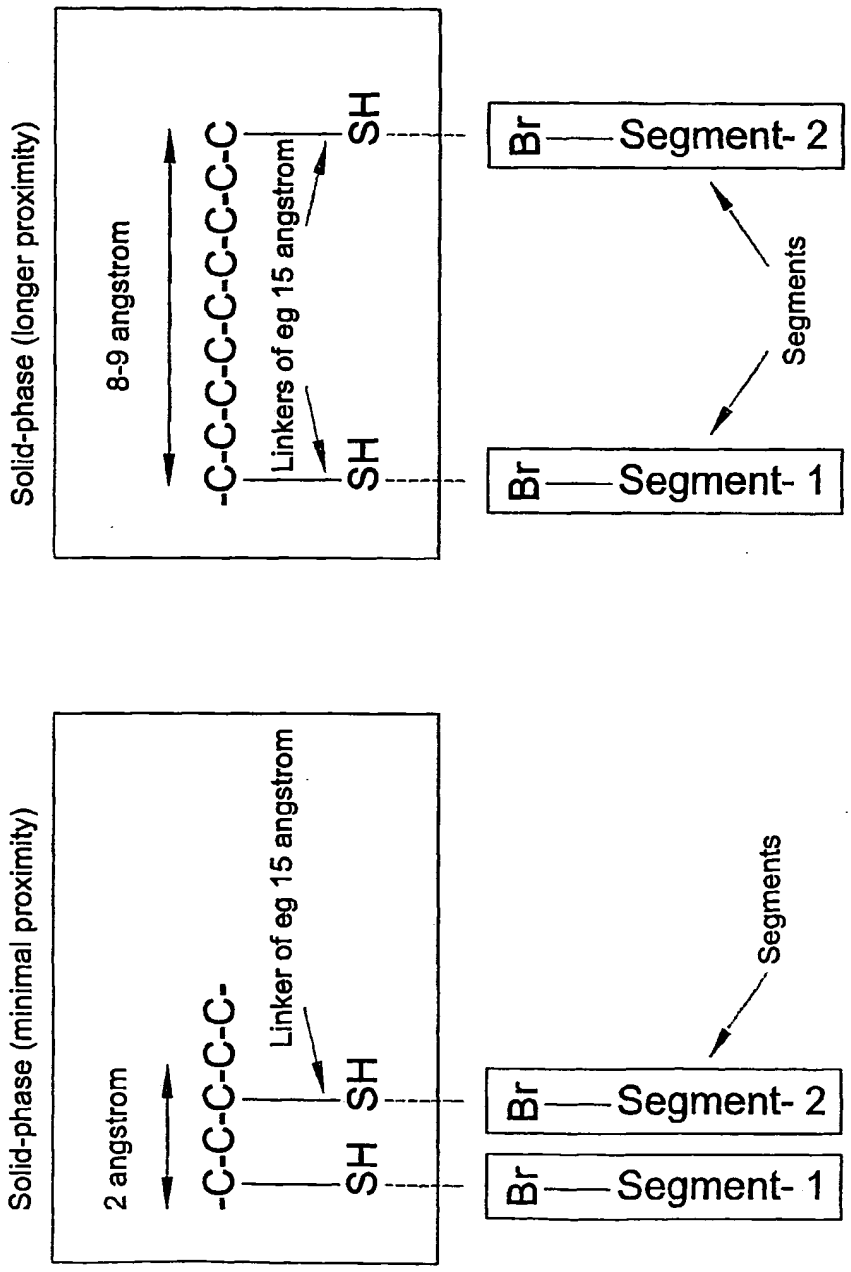

Solid-phase (longer proximity)
8-9 angstrom
-C-C-C-C-C-C-C-C-C
Linkers of eg 15 angstrom
SH
SH
Br
Segment- 2
Segments
Br
Segment- 1
Solid-phase (minimal proximity)
2 angstrom
-C-C-C-C-C-
Linker of eg 15 angstrom
SH
SH
Segments
Br
Segment- 2
Br
Segment- 1

Fig. 3

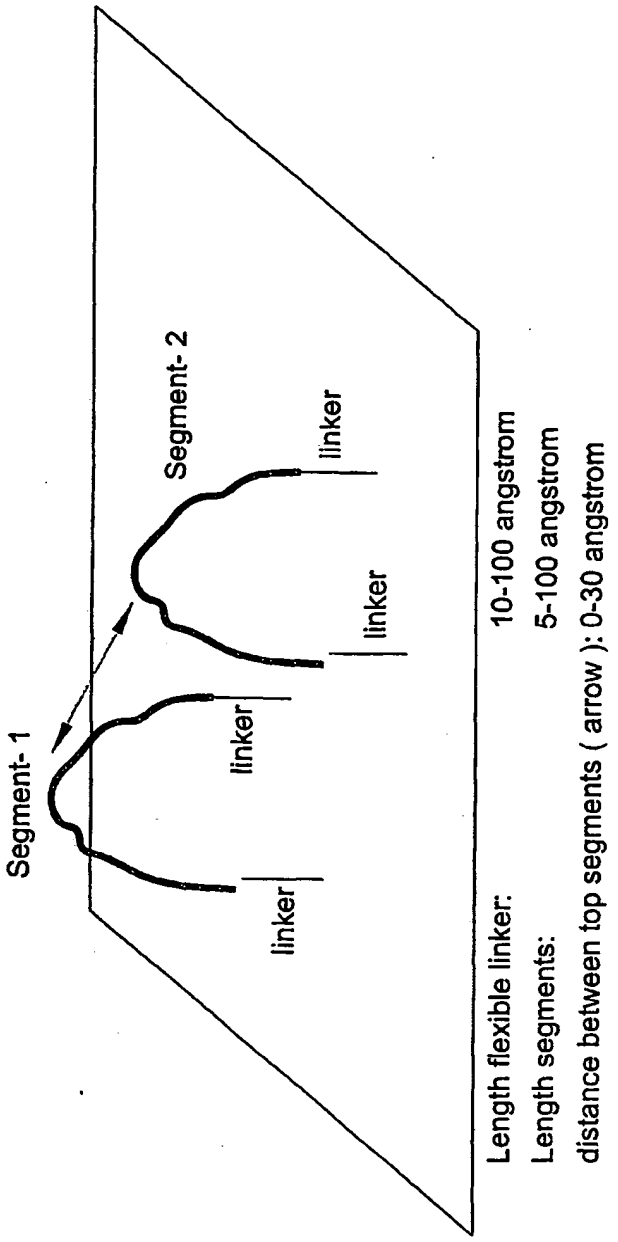

Segment- 1
Segment- 2
linker
linker
linker
linker
Length flexible linker: 10-100 angstrom
Length segments: 5-100 angstrom
distance between top segments (arrow): 0-30 angstrom

Fig. 3B

Fig. 4

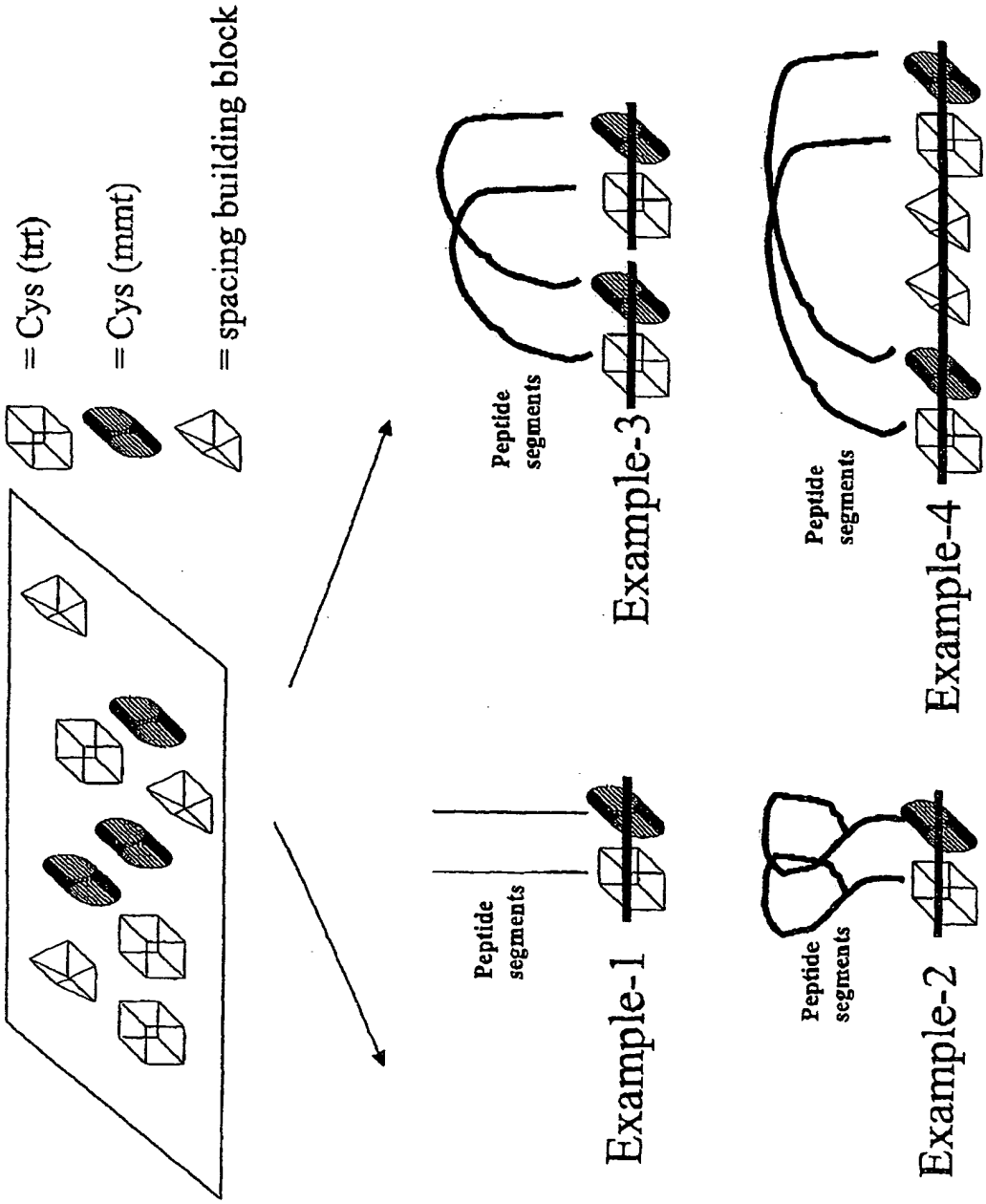

= Cys (trt)
= Cys (mmt)
= spacing building block
Peptide segments
Example-1
Peptide segments
Example-2
Peptide segments
Example-3
Peptide segments
Example-4

Fig. 4B

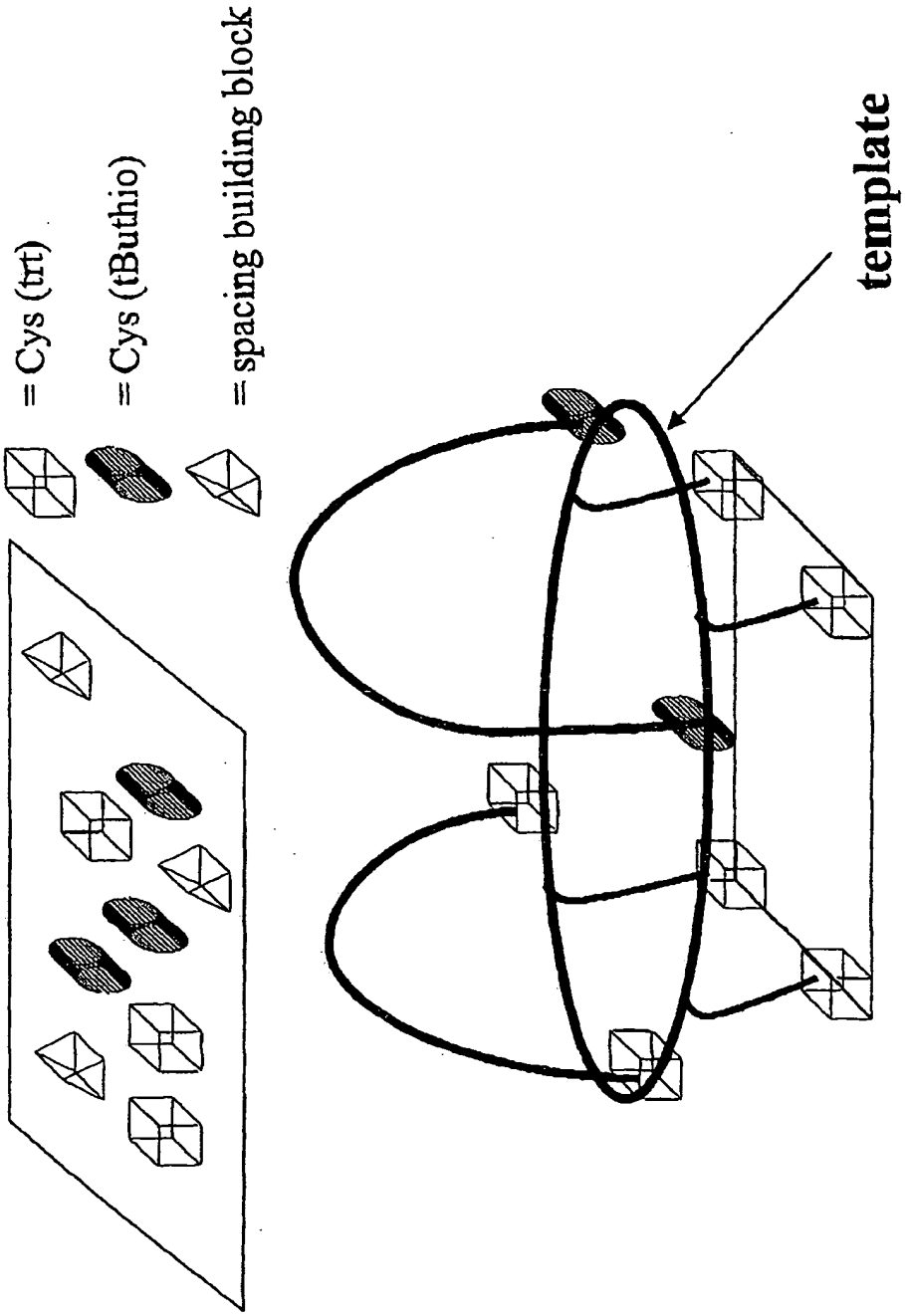
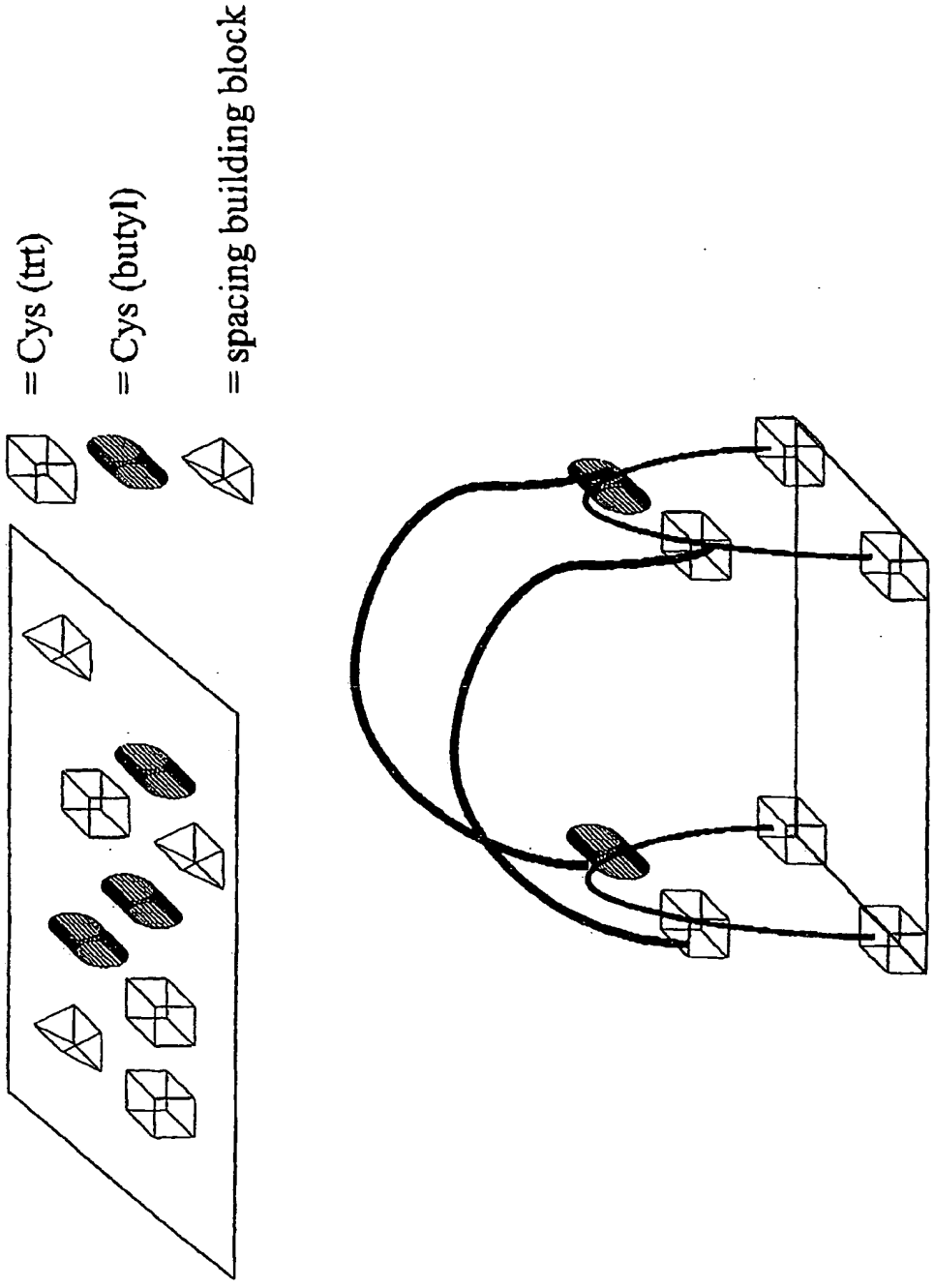


Fig. 4C



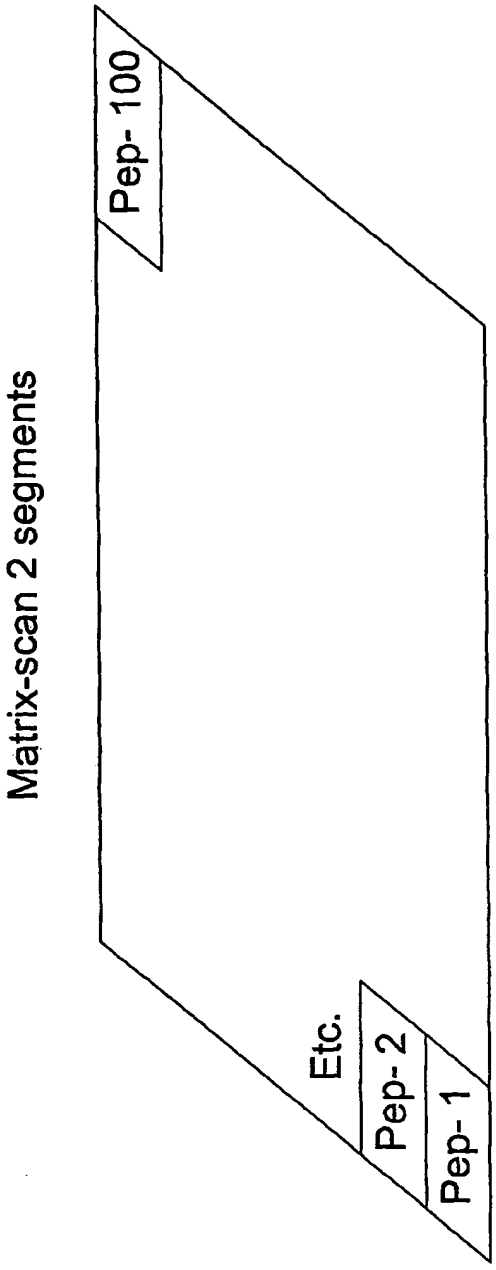


Fig. 5

Fig. 6

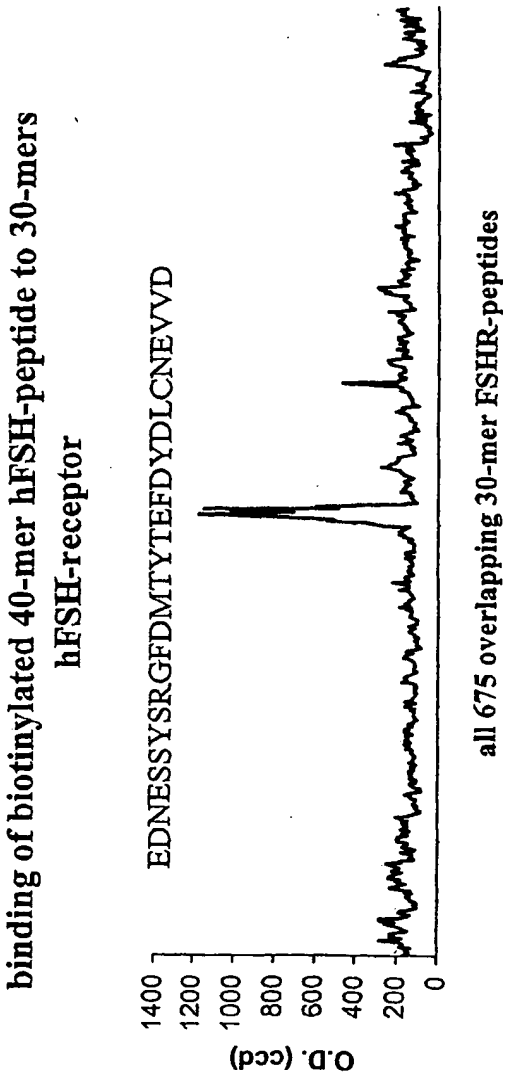

binding of biotinylated 40-mer hFSH-peptide to 30-mers
hFSH-receptor
EDNESSYSRGFDMTYTEFDYDLCNEVVD
O.D. (ccd)
1400
1200
1000
800
600
400
200
0
all 675 overlapping 30-mer FSHR-peptides

Fig. 7

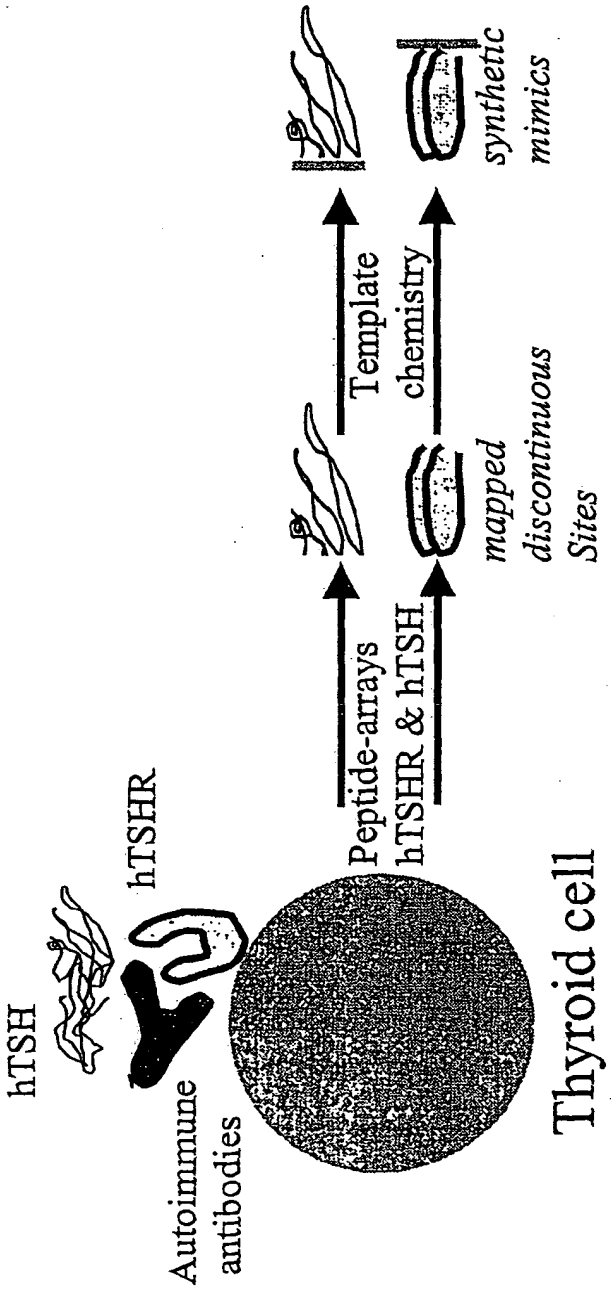

hTSH
hTSHR
Autoimmune antibodies
Thyroid cell
Peptide-arrays hTSHR & hTSH
Template chemistry
mapped discontinuous Sites
synthetic mimics

Fig. 8

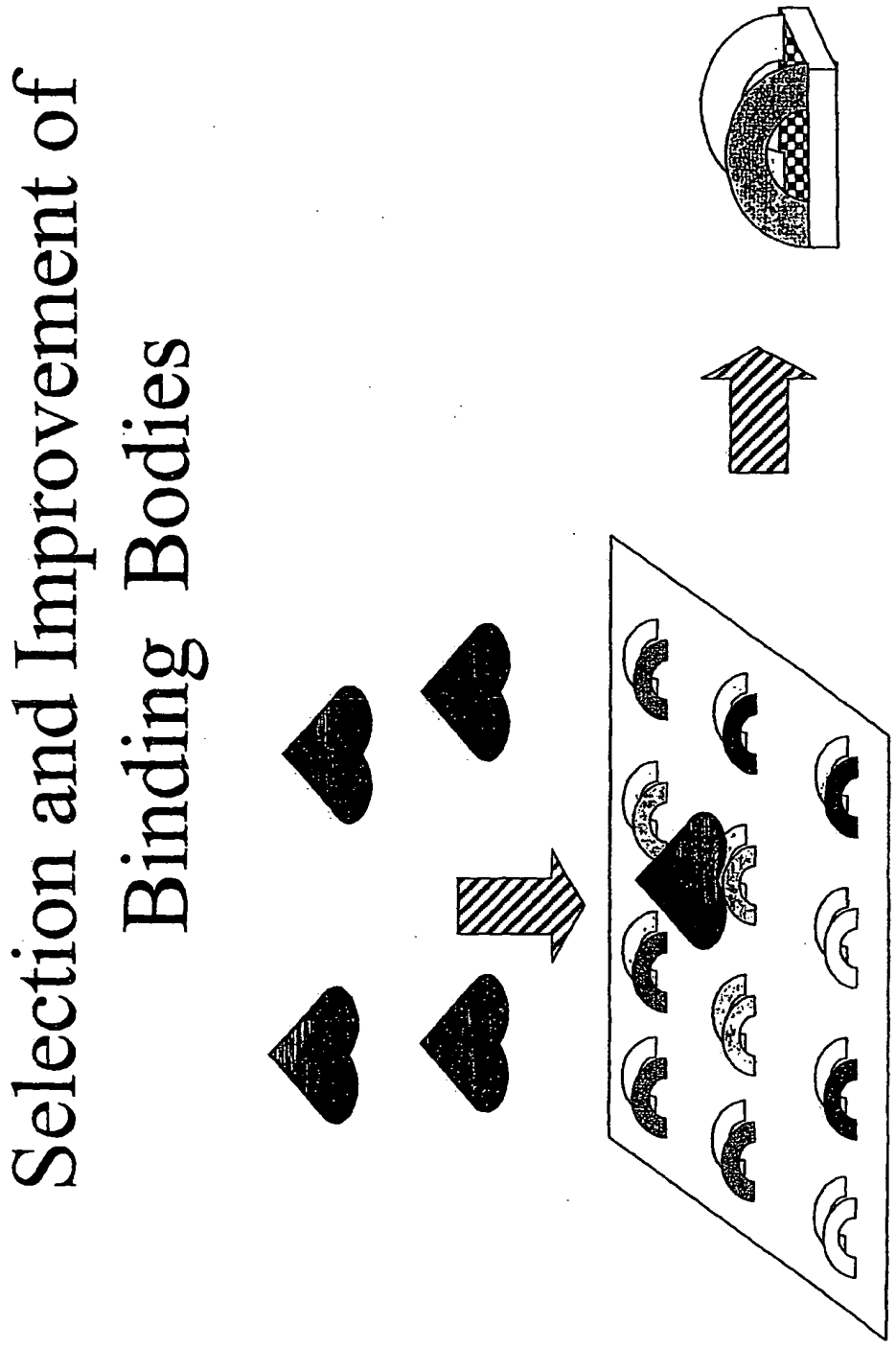


Fig. 9

Binding Bodies

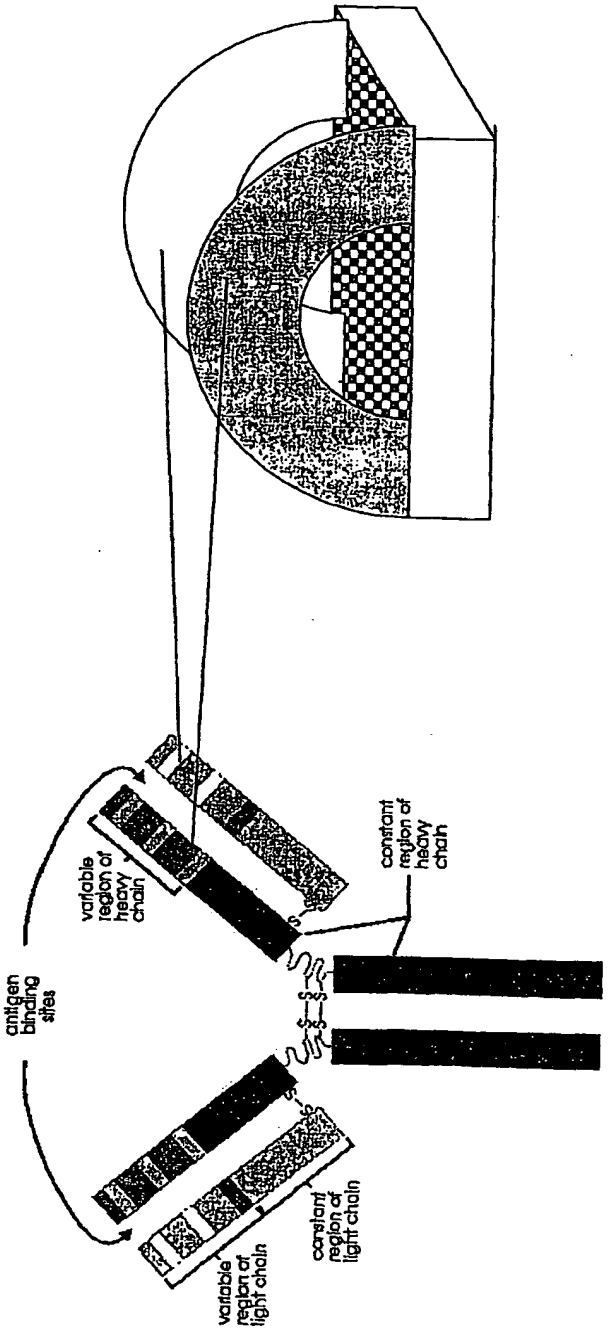


Fig. 10

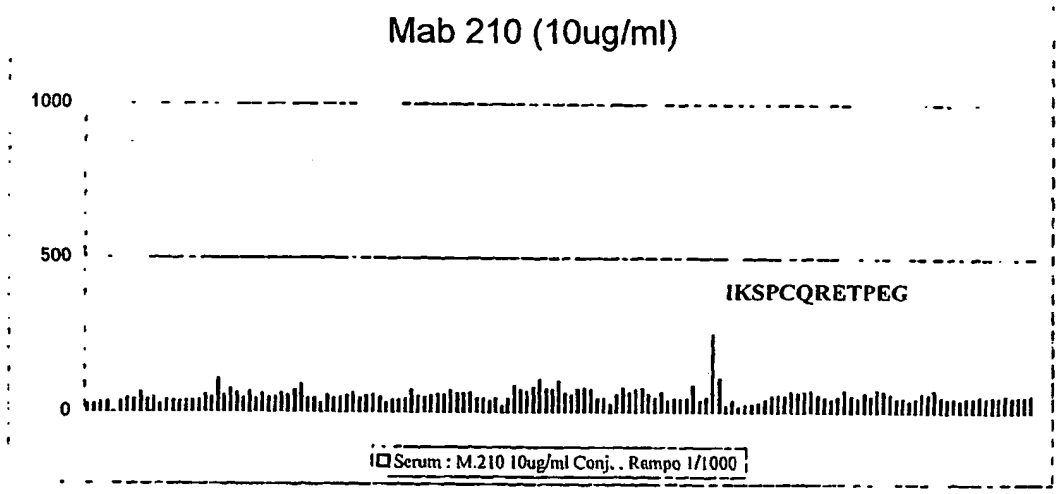

Mab 210 (10ug/ml)
1000
500
0
IKSPCQRETPEG
Serum : M.210 10ug/ml Conj. . Rampo 1/1000

Fig. 11

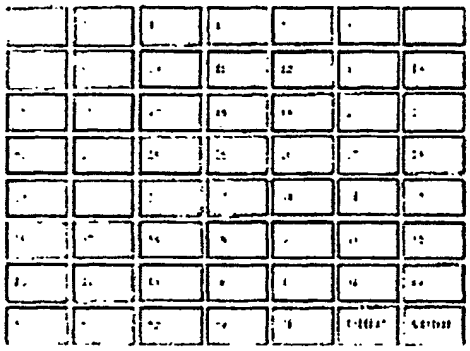
1. +AVRSSSRTPSDKPVZ
2. +VRSSSRTPSDKPVAZ
3. +RSSSRTPSDKPVAHZ
4. +SSSRTPSDKPVAHVZ
5. +SSRTPSDKPVAHVVZ

Etc.

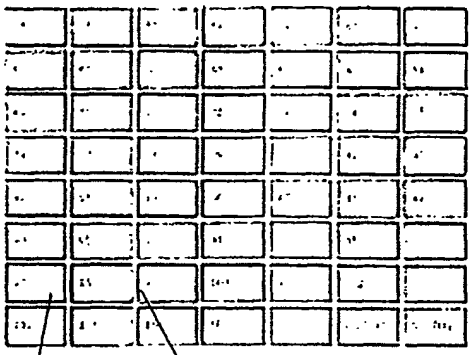
145. +FAESGQVYFGIIALZ

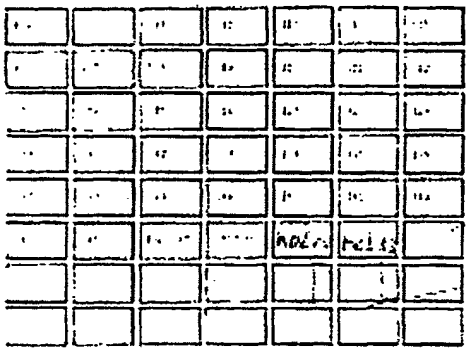
Fig. 12

Grid-44



Grid-46





Grid-48

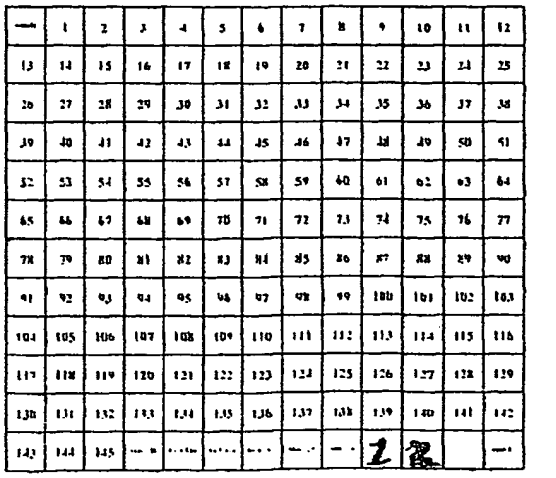


Fig. 13

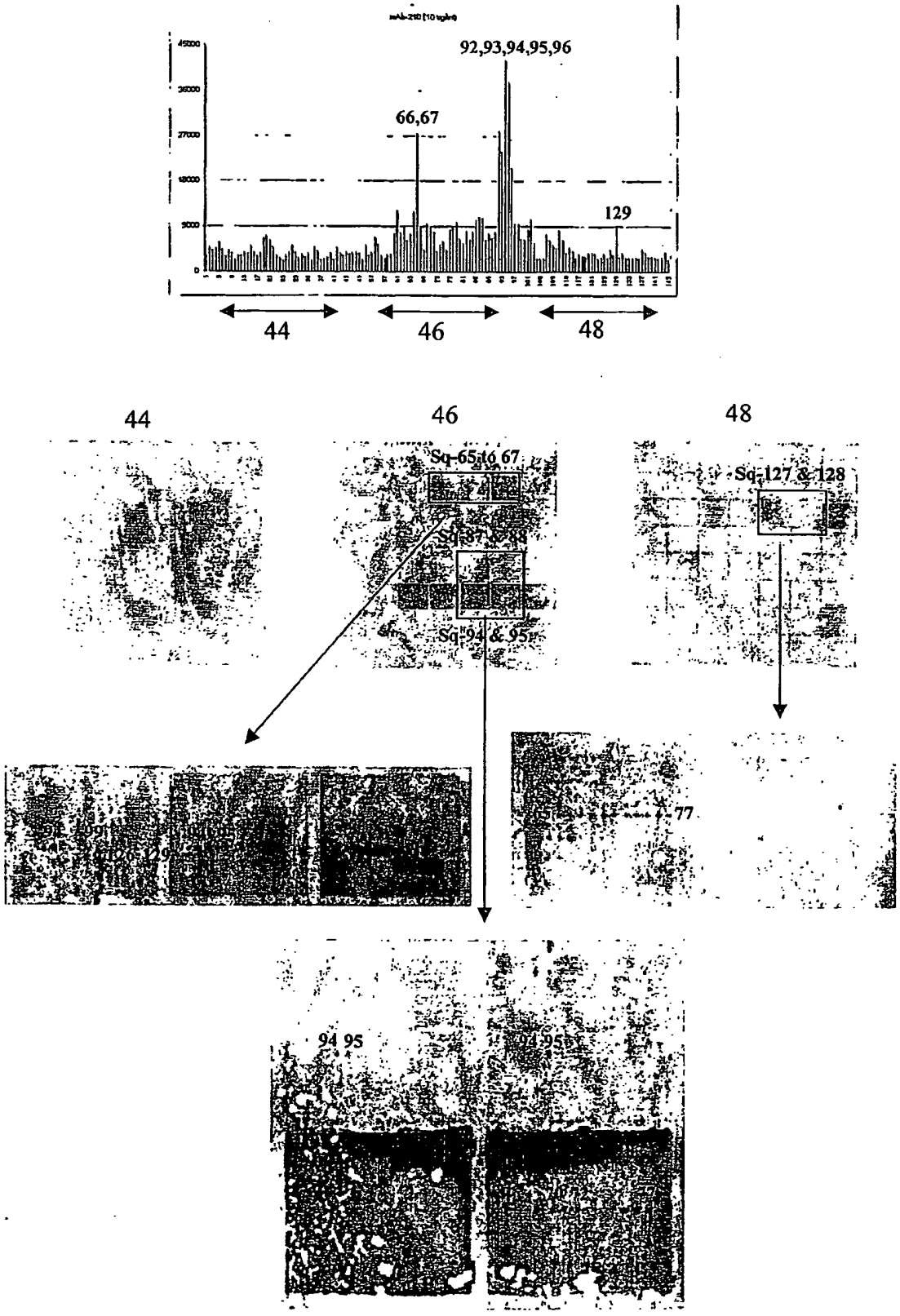
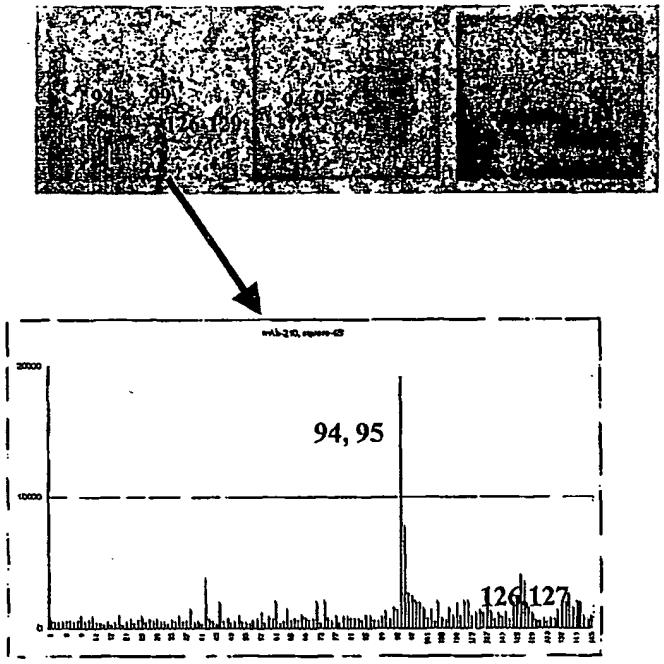

92,93,94,95,96
66,67
129
44
46
48
44
46
48
Sq 65,66 67
Sq 87 & 88
Sq 94 & 95
Sq 127 & 128
77
94 95
94 95

Fig. 14

65



127

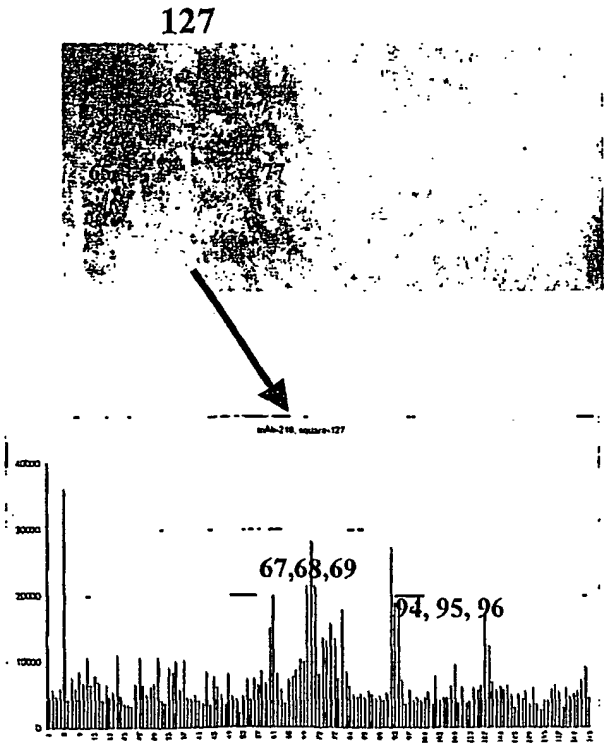
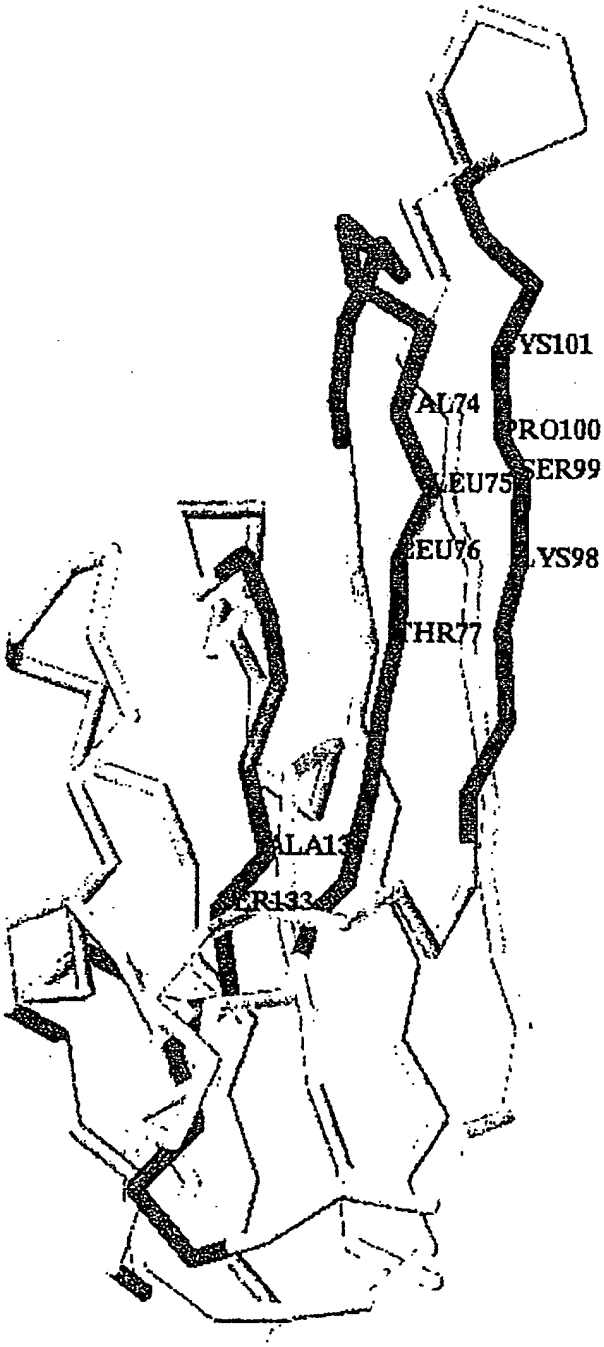


Fig. 15



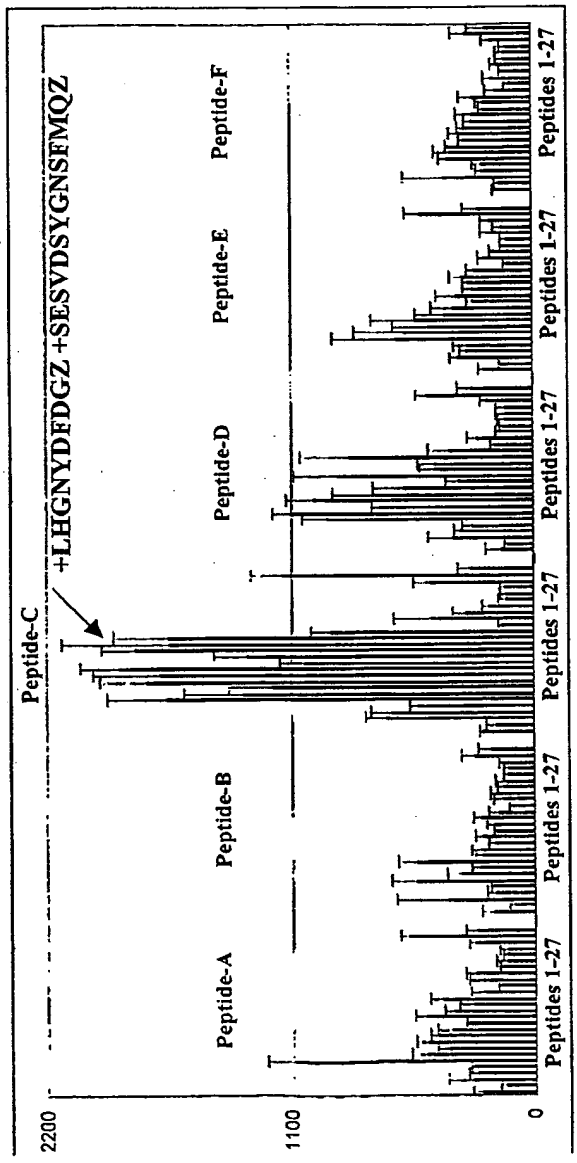
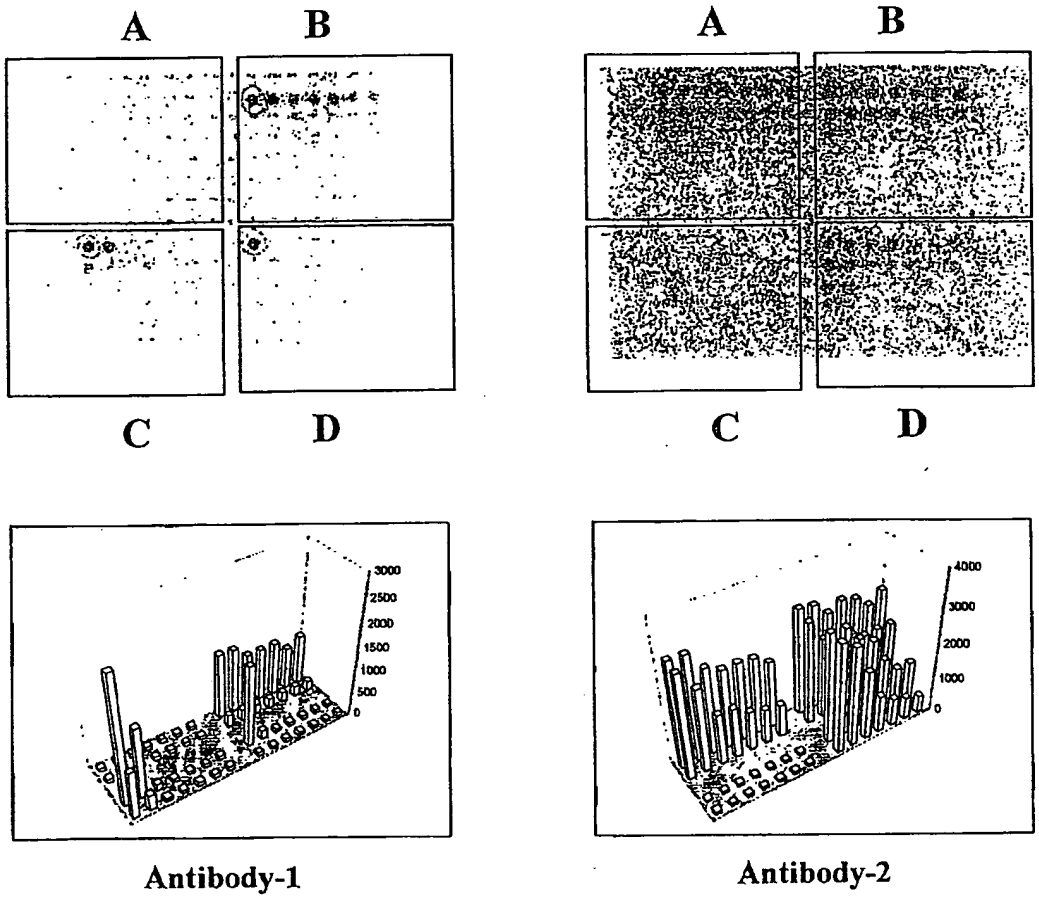

Peptide-C
+LHGNYDFDGZ +SESVDSYGNSFMQZ
Peptide-A
Peptide-B
Peptide-D
Peptide-E
Peptide-F
Peptides 1-27
2200
1100
0

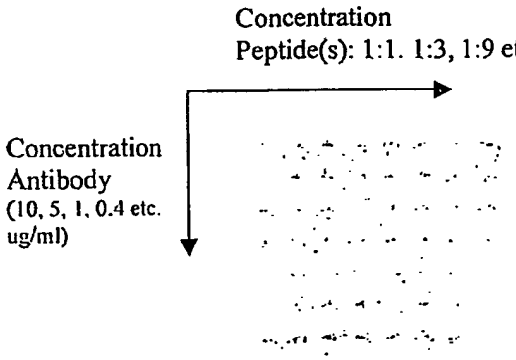
Fig. 16

Fig. 17



Antibody-1

Antibody-2



INTERNATIONAL SEARCH REPORT

Internat Application No
PCT/NL 01/00744

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/68 C07K1/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	REINEKE ULRICH ET AL: "A synthetic mimic of a discontinuous binding site on interleukin-10." NATURE BIOTECHNOLOGY, vol. 17, no. 3, March 1999 (1999-03), pages 271-275, XP002168865 ISSN: 1087-0156 cited in the application the whole document	1-12,16, 17
A	WO 97 00267 A (PENCE INC) 3 January 1997 (1997-01-03) the whole document	1-12,16, 17
A	WO 96 09411 A (CYTOGEN CORP) 28 March 1996 (1996-03-28) the whole document	1-12,16, 17

-/--

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 December 2001	13/02/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P B 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel (+31-70) 340-2040, Tx 31 651 epo nl, Fax (+31-70) 340-3016	Pellegrini, P

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INTERNATIONAL SEARCH REPORT

Interna - Application No
PCT/NL 01/00744

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	WO 84 03564 A (COMMW SERUM LAB COMMISSION) 13 September 1984 (1984-09-13) cited in the application the whole document -----	1-12,16, 17

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat application No
PCT/NL 01/00744

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		WO 9609411 A1	28-03-1996
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		US 5595915 A	21-01-1997

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 13-15,18

Claims 13-15 and 18 relate to a molecule defined by reference to a desirable characteristic or property, namely being identified by the screening method of claims 11-12.
The claims cover all molecules having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for no such molecules.
In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the subject-matter for which protection is sought in claims 13-15 and 18 is impossible. Consequently, no search has been performed on these claims.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.